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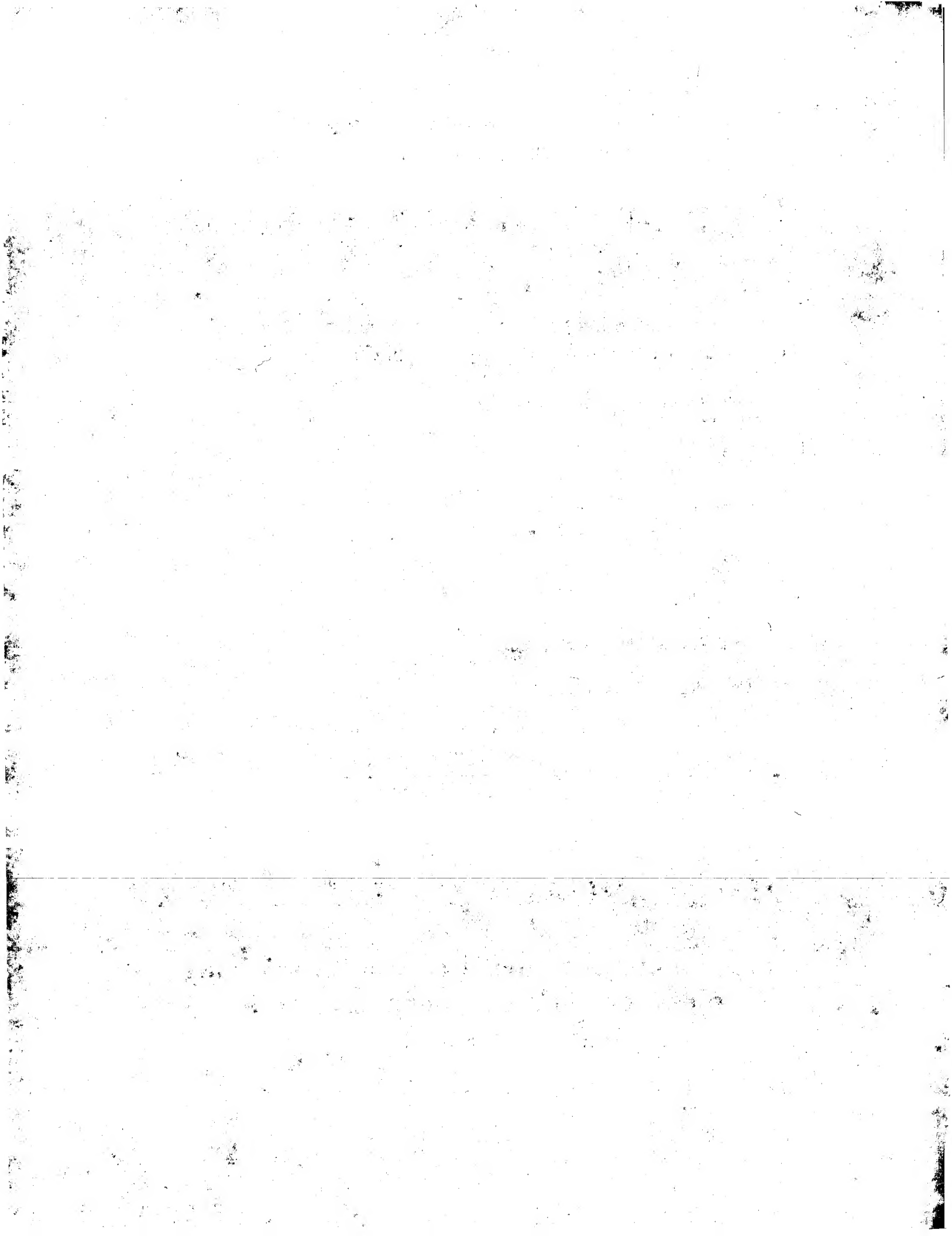
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(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) P54 Stress-Activated Protein Kinases

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p54 STRESS-ACTIVATED PROTEIN KINASES

Abstract of the Disclosure

The p54 Stress-Activated Protein Kinases (SAPKs) are members of a large gene family and are structurally related to the previously described mitogen-activated protein kinases (MAPKs). They are distinguished by their strong activation in response to heat shock, TNF- α , IL-1- β , sphingomyelinases, chemical protein synthesis inhibitors, and ischemia. These kinases have potential utility in the modulation of the inflammatory response and the up-regulation of repair or protective cellular proteins following injury or chemical insult.

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p54 STRESS-ACTIVATED PROTEIN KINASESStatement as to Federally Sponsored Research

5 This invention was funded at least in part by the following grants from the United States Government: GM46577 to JMK, DK17776 and DK41513 to JA (DK41513 transferred to JMK as of 1/94), and the U.S. Government has certain rights in the invention.

10 Background of the Invention

The field of the invention is second messenger protein kinases which regulate activation of transcription factors, which in turn modulate cellular responses to extracellular stimuli.

15 The extracellular signal-regulated kinases (ERKs) are a family of proline-directed kinases that are activated via concomitant Tyr and Thr phosphorylation and share sequence homology in the Ser/Thr protein kinase catalytic domain. They include the mitogen activated
20 protein kinases, or MAPKs. Stimuli for the activation of these kinases are diverse, and induce discrete second messenger pathways to effect specific cellular responses. ERKs participate in the regulation of other protein kinases and several transcription factors including c-
25 Jun, c-Myc, c-Fos, ATF-2, and p62^{TCF}/Elk-1. These functions indicate that the ERKs mediate the expression of genes in response to extracellular agonists.

The first well characterized members of this kinase family were p42 and p44 MAPKs, which are
30 stimulated by insulin and require both Tyr and Thr phosphorylation for activity (Sturgill et al, Nature 334:715-718, 1988; Anderson et al., Nature 343:651-653, 1990). They are also stimulated by a variety of mitogens, phorbol esters, and activated ras.

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The ERKs share sequence homology in the Ser/Thr protein kinase catalytic domain, as mentioned above, and this functions to phosphorylate c-Jun on serine and threonine residues that have been localized to c-Jun tryptic peptides termed X and Y. X and Y are located near the N-terminal trans-activation domain (Pulverer et al., *Nature* 353:670-673, 1992). Phosphorylation of these peptides regulates transactivating binding activity, and thus their function as promoters of gene expression.

10 Summary of the Invention

The invention features a molecule consisting of either DNA or amino acids encoding a p54 stress-activated protein kinase (SAPK), or a biologically active fragment thereof, characterized by its ability to modulate transcription pathways in response to extracellular stress stimuli. By "biologically active fragment" it is meant that the fragment can exert a physiological effect (e.g., binding to its biological substrate, phosphorylation, causing an antigenic response, etc.) in vivo or in vitro.

In preferred embodiments, the molecule of the invention is a polypeptide at least 95% identical to p54 α I, p54 α II, p54 β I, p54 β II, or p54 γ , preferably from a mammalian source (e.g., SEQ ID NOs: 1, 2, 3, 4, or 5). Any polypeptide sequence containing an "X" is intended to denote a position that could be any amino acid. By "polypeptide", it is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

30 Other preferred embodiments include polypeptides that are substantially identical to SEQ ID NOs 1, 2, 3, 4, 5, 6, 7, and 8. By "substantially identical", it is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same

class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy
5 the biological function of the polypeptide.

In one preferred embodiment, the molecule of the invention is a polypeptide fragment that contains a biologically active portion of a p54 polypeptide, at least 10 amino acids in length. Examples include, but
10 are not limited to, the ATP binding site, which includes Lys₃₅ and Gly_{33, 35, and 38}; the site of regulatory phosphorylation by upstream activators (Thr₁₈₃-Pro-Tyr₁₈₅); and the SAPK catalytic domain (between amino acids 22 and 321; see Fig. 1).

15 In another preferred embodiment the polypeptide or a fragment thereof is useful for producing antibodies which specifically bind to a p54 stress-activated protein kinase (e.g., SEQ ID NOs 6, 7, and 8). In this context, fragment means at least the smallest antigenic epitope,
20 generally at least 10 contiguous amino acids.

The invention also features a DNA molecule encoding a p54 stress-activated protein kinase polypeptide, its degenerate variants, or a fragment thereof including at least 30 contiguous nucleotides.
25 The DNA sequence may be 90% identical to p54 α I (e.g., SEQ ID NO: 9), p54 α II (e.g., SEQ ID NO: 10), p54 β I (e.g., SEQ ID NO: 11), p54 β II (e.g., SEQ ID NO: 12), or p54 γ (e.g., SEQ ID NO: 13), or be a fragment of any of the above nucleotide sequences containing at least 30 contiguous
30 nucleic acids, or a degenerate variant thereof. By "degenerate variant" it is meant any nucleotide sequence that encodes the same amino acid sequence as the polypeptide translated from the DNA, or a substantially identical polypeptide.

The molecules of the invention are preferably derived from a mammal, more preferably from a rat or human.

The invention also includes DNA molecules which
5 hybridize under stringent conditions to one or more of the DNAs encoding p54 SAPKs (SEQ ID NOs 9, 10, 11, 12, and 13). By "stringent conditions" it is meant conditions under which molecules without significant
10 homology (e.g., at least 90%) to the DNAs of the invention could not hybridize (protocols to determine stringency and melting point of DNA sequences are well known to those skilled in the art and may be found in Sambrook et al. (eds), Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, 1989; hereby
15 incorporated by reference). Included in the term "DNA(s)" are the sequences of both strands of double stranded DNA.

The invention also features methods of screening potentially therapeutic compounds involving applying test
20 compounds in physiologically relevant concentrations in a suitable excipient to cultured cells, with or without previous, prior, or concurrent stress-activated protein kinase-activating stimuli (e.g., TNF- α , IL-1- β , sphingomyelinase, heat shock, etc.), preparing cellular
25 extracts, and assaying the isolated recombinant stress-activated protein kinase for c-Jun kinase activity. By "physiologically relevant" it is meant a concentration that is achievable during non-toxic administration to a human patient. By suitable excipient it is meant a non-
30 toxic or non-injurious solvent or carrier for the molecules of the invention or test compounds used in the screening assay.

In another embodiment, the cells treated with the test compound and with or without previous, prior, or
35 concurrent SAPK activating stimuli are extracted, and

combined with inactive recombinant stress-activated protein kinase. The recombinant SAPK is then isolated and assayed for c-Jun kinase activating ability.

The advantages and uses of the invention are in
5 the treatment and prevention of inflammation and the deleterious effects of hypoxia, heat stress, reperfusion injury, and other tissue insults which are currently difficult to manage clinically. The molecules of the invention may be useful for reducing inflammation in such
10 chronic disorders as autoimmune diseases or allergies, and in acute conditions such as anaphylactic shock, or soft tissue injury where swelling may aggravate the condition (e.g., around broken bones). Other uses include prophylactic treatment of patients about to undergo
15 surgeries where there is a high likelihood of ischemia-reperfusion injury (e.g., vascular surgery, organ transplants, etc.), or treatment of sepsis and fever. Additionally, these molecules could be used to up-regulate AP-1 expression via c-Jun phosphorylation, and
20 thus enhance levels of IL-2 expression as a cancer therapeutic.

The molecules of the invention may also be useful for drug design based on an understanding of the enzymes' structure and function (e.g., the polypeptide's binding
25 site on c-Jun), or as indicators in an assay to screen large numbers of drugs for beneficial effects on the conditions listed above.

Other features and advantages of the invention will be apparent from the following description of the
30 preferred embodiments, and from the claims.

Brief Description of the Drawings

Figure 1: Deduced amino acid sequences (SEQ ID NOS: 1, 2, 3, 4, 5, 6, 7, and 8) alignment of p54 stress-activated protein kinase (SAPK) cDNAs (single

letter code). Sequences determined by protein microsequencing are underlined, and the black diamonds flank the catalytic domains of the SAPKs.

Figures 2 a) and b): Specificity of anti p54 SAPK antisera for p54 SAPKs. a) In vitro translation of ERKs. RNAs (2 μ g) transcribed from plasmids containing the cDNAs of rat p54 α I (lane 1), p54 β (lane 2), and HA-epitope-tagged p44 MAPK (lane 3) were translated using rabbit reticulocyte lysates. Lane 4 shows lysate programmed with water; a 43-kDa background band is indicated by an open arrowhead. b) Aliquots of the translated proteins were immunoprecipitated in RIPA buffer with antisera raised against bacterially expressed p54 β . Lanes are the same as in the left panel (lanes 1, 2, and 3).

Figures 3 a) and b): HepG2 cells were treated with TNF- α (100ng/ml, 15 min). Cell extracts were prepared and depleted of SAPKs by exhaustive immunoprecipitation for the times indicated (a) or for 4 hrs (b), at which time extracts were subjected to GST-c-Jun chromatography as a means of determining the c-Jun kinase activity remaining in the extracts. As a control, immunodepletions were done with preimmune serum or without serum (b). Activity in the immunoprecipitates and on the c-Jun columns was measure (a). The results indicate that around 70% of the c-Jun kinase activity present in the extracts can be removed with the anti-SAPK serum.

Figures 4 a) and b): Comparative activation of p42/44 MAPKs (open bars) and p54 SAPKs (filled bars) showing that NIH3T3 cell (a) and HT-29 cell (b) p54 SAPKs are poorly activated by mitogens and strongly activated by heat stress and cycloheximide.

Figure 5 a) and b): Activation of c-Jun phosphorylation in situ by various stimuli. a) c-Jun phosphorylated in vivo in response to cycloheximide (lane

1) and heat shock (lane 2); retardation of the Jun polypeptide upon SDS-PAGE (compare lanes 1 and 2 with non-phosphorylated c-Jun in lane 3). The c-Jun polypeptide is indicated with an arrowhead. b) Two dimensional tryptic phosphopeptide mapping of c-Jun polypeptides immunoprecipitated from control (left), or heat shock-treated (right) HepG2 cells indicates enhanced phosphorylation of peptides X and Y in response to heat shock. The origin is marked with an arrowhead. The dotted circle indicates the position of the xylene cyanol marker.

Figures 6 a) and b): Detection and isolation of c-Jun kinases activated by heat shock and other stimuli on GST-c-Jun immobilized on glutathione agarose (b); comparison with activation of p54 SAPKs (a). The black and white bars each represent results from one experiment.

Figures 7 a) and b): Activation of SAPKs and c-Jun kinases by tunicamycin. p54 SAPK activity is shown in a, closed circles (mean \pm SD for triplicate determinations), total c-Jun kinases binding to immobilized GST-c-Jun, a, open circles; b shows the fold activation of the p42/p44 MAPKs by tunicamycin.

Figure 8 a) and b): Comparison of activation of p54 SAPKs and p42/44 MAPKs by various stimuli in human CCD-18Co cells. a) Activation of p54 SAPKs by TNF- α and other stimuli. Mean \pm SD for triplicate determinations is shown. b) Parallel assays for relative activation of p42/44 MAPKs by the same stimuli shown in a).

Figure 9 a) and b): Comparison of activation of p54 SAPKs and p42/44 MAPKs by various stimuli in primary porcine hepatocytes. a) Activation of p54 SAPKs by TNF- α and other stimuli. Mean \pm SD for triplicate determinations is shown. b) Comparison of fold

activation by various stimuli of p54 SAPKs (filled bars) and p42/44 MAPKs (open bars).

Figure 10: Activation of HepG2 cell p54 SAPKs by TNF- α and *S. aureus* sphingomyelinase. SMase = sphingomyelinase.

Figures 11 a) and b): Comparison of activation of p54 SAPKs and p42/44 MAPKs by IL-1- β . EL-4 murine thymoma cells were treated with 20 ng/ml recombinant human IL-1- β . Cells were extracted as described in the legend of Table 2. SAPKs (a and filled bars, b) and p42/44 MAPKs (b) were assayed in standard experimental paradigms. For a) data are mean \pm SD for triplicate determinations. For b) data are presented as percent of control for comparative purposes.

Figures 12 a) and b): Effect of ischemia/reperfusion on MAPK and SAPK activation *in vivo*. Activation of p54 SAPK (a) and p42/44 MAPKs (b) was measured at times from 0-90 min after initiating reperfusion of rat kidneys made ischemic for 45 min. as described in the Methods section. MAPKs experienced rapid activation and inactivation (b), while SAPKs were rapidly activated and remained at elevated levels for periods well in excess of 90 min.

Figure 13: Bacterially expressed rat SAPK- α was expressed as a GST fusion protein and purified on glutathione agarose. The SAPK was treated with buffer alone, or with an extract from HepG2 cells treated with TNF and prepared as described in the Table 2 legend. The SAPK was recovered with glutathione beads and assayed for c-Jun kinase activity. As an additional control, blank beads were exposed to cell extracts (extract alone bar).

Description of the Preferred EmbodimentsMethodsCloning

p54 SAP kinase was purified to homogeneity from
5 livers of cycloheximide injected rats (Kyriakis and
Avruch, *J. Biol. Chem.* 265:17355-17363) and the sequences
of several tryptic peptides were determined following RP-
HPLC. Two of these peptides (HRDLKPSN and
MLVIDPDKRISVDEAL) were homologous to protein kinase
10 subdomains VIb and XI, respectively (Hanks et al.,
Science, 241:42-52, 1988) and were used to design
degenerate sense and antisense primers, respectively. A
467 bp fragment was amplified by PCR from rat brain cDNA
and used as a probe to screen 250,000 plaques of a rat
15 brain cDNA library in λ ZAP (Stratagene). Twenty seven
positive plaques were purified and representatives
sequenced on both strands using nested deletion series.

Antisera/Antibodies

To generate antisera, the p54 SAPK- β isoform was
20 subcloned into pGEX-KG and expressed as a glutathione S-
transferase (GST) fusion protein. Expression p54 SAPK- β
polypeptide was induced with IPTG (50 μ g/ml) and the
fusion purified to homogeneity by glutathione-agarose
chromatography, followed by thrombin cleavage to release
25 the kinase moiety from the fusion protein. This material
was then used as an immunogen and antisera collected and
tested. The antisera tested have been shown to cross-
react with human, mouse, rat, and pig tissues. The SAPK
sera react with several human cell lines, including human
30 hepatocellular carcinoma cells (HepG2), human colon
fibroblasts (CCD-18Co), human monocytic lymphoma cells
(U937), and human colon carcinoma cells (HT-29).

Monoclonal antibodies may be made by fusing immune
B cells from the spleen with tumor cells to produce

hybridomas specifically secreting each antibody, using methods well known in the art (see, for instance, Coligan et al., eds. Current Protocols in Immunology, John Wiley and Sons, 1992; Kohler et al., *Nature* 256:495, 1975; 5 Hammerling et al., in Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981)

Peptide antisera were generated using standard methods to the p54 α , p54 β , and p54 γ classes using the least conserved region of the molecules (SEQ ID NOS: 6, 10 7, and 8) to enhance the probability of class-specificity.

Cell Culture Treatments and Assay

Confluent cultures of NIH3T3 cells were treated with H₂O₂ (5 mM, 15 min) phorbol-12-myristate-13-acetate 15 (PMA, 500 nM, 20 min) FGF (10 ng/ml, 20 min), A23187 (100 nM 20 min), heat (42°C, 30 min) or cycloheximide. HT-29 cells were treated with EGF (50 ng/ml, 20 min) or heat shock (42°C, 30 min). Cells were washed three times in PBS and lysed in ice-cold lysis buffer (20 mM Na- 20 Hepes, pH 7.5, 2 mM EGTA, 1 mM DTT, 1 mM Na₃VO₄, 50 mM β -glycerophosphate, 1% (w/v) Triton X-100, 10% (v/v) glycerol, 2 μ M leupeptin, 10 kallikrein-inhibiting units/ml aprotinin, 200 μ M PMSF). Extracts were normalized to identical protein concentration (1 mg/ml). 25 A portion (1 ml) was immunoprecipitated with 5/13-99, and assayed for GST-Jun kinase activity as follows. To 40 μ l of a 1:1 suspension of p54 SAPK beads were added 20 μ l 0.2 mg/ml GST-c-Jun-1-135 (Adler et al., (1992), *Proc. Natl. Acad. Sci. U.S.A.*, 89:5341-5345) or 0.01 mg/ml 30 holo-c-Jun (Pulverer et al., *supra*; Pulverer et al., (1993), *Oncogene*, 8:407-415). ATP (100 μ M) and MgCl₂ (10 mM) were added to start the reaction. The reaction was allowed to proceed for 20 min at 30°C at which time the reaction was stopped with SDS sample buffer and the

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mixtures resolved by SDS-PAGE. The 40-kDa GST-Jun band was excised and counted by liquid scintillation spectroscopy (mean \pm SD for triplicate determinations are shown in the figures). Another portion (1 ml) was
5 assayed for p42/p44 MAPK activity by Mono-Q chromatography, and by a myelin basic protein (MBP) kinase activity assay (Ahn et al., (1991), *J. Biol. Chem.*, 266:4220-4227). A peak of stimulated MBP kinase activity was always detected eluting between 200 and 350
10 nM NaCl. Total p42/p44 MAPK activity was taken as that contained in those fractions and is shown as percent of control for comparative purposes.

U937 cells were labeled with ^{32}P orthophosphate (1 mCi/ml) and treated with cycloheximide heat stress or
15 vehicle. c-Jun was immunoprecipitated using a polyclonal antibody specific for the C-terminal 15 amino acids of c-Jun and subjected to SDS-PAGE (Pulverer et al.). HepG2 cells were labeled with ^{32}P as above and subjected to heat shock. c-Jun was immunoprecipitated and, after SDS-PAGE,
20 was subjected to two dimensional tryptic phosphopeptide mapping. In other experiments, U937 cells were treated with actinomycin-D (10 $\mu\text{g/ml}$, 30 min), anisomycin (10 $\mu\text{g/ml}$, 30 min) emetine (10 $\mu\text{g/ml}$, 30 min), puromycin (10 $\mu\text{g/ml}$, 30 min) or heat stress (42°C, 30 min). Lysates
25 were prepared and were passed over columns of immobilized GST-Jun. After washing away unbound proteins, $\text{Mg}/\gamma\text{-}^{32}\text{P}\text{-ATP}$ was added and phosphorylation of the immobilized c-Jun was assayed as described above. Parallel aliquots were subjected to immunoprecipitation and assayed for p54
30 SAPK. In both assays, 1 U of kinase activity transferred 1 pmol PO_4/min from ATP to GST-Jun. HT29 cells were treated with various concentrations of tunicamycin for 5 h. Lysis, assay of p42/p44 MAPK activity, and p54 SAPK immune complex kinase assay were done by standard
35 methods. Detection of total tunicamycin-stimulated c-Jun

kinase activity in HT-29 cells isolated on columns of immobilized c-Jun was performed as described above.

- CCD-18Co cells, HepG2 cells, or primary porcine hepatocytes were cultured to 80% confluence, serum starved (0.5% serum, 16 hours) and treated with the following agonists as shown in Figures 16 and 17: heat shock (42°C, 30 min), PMA (200 nM, 20 min), EGF (100 ng/ml, 20 min) or TNF- α (50 ng/ml, 10 or 20 min). Extracts were prepared and p54 SAPKs were immunoprecipitated and assayed. Parallel assays of p42/p44 MAPKs were performed. For treatment with sphingomyelinase, 100 mU/ml *S. aureus* sphingomyelinase were added to HepG2 cells as known in the art (Dressler et al., (1992), *Science*, 255:1715-1718) for 15 min. *S. aureus* sphingomyelinase hydrolyzed 1 μ mole TNP-sphingomyelin/min at pH 7.4, 37°C.

Ischemia/Reperfusion

- Male Sprague-Dawley rats, 120-150 g each, were anesthetized with sodium pentobarbital (65 mg/kg). In order to induce ischemia, the renal artery of one kidney was clamped for 45 min. The contralateral kidney served as a control. Reperfusion was accomplished by releasing the clamp and allowing blood flow for 0 to 90 min. Control and ischemia/reperfusion kidneys were harvested and homogenized in lysis buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 50 mM β -glycerophosphate, 1 mM DTT, 250 mM sucrose, 400 μ M PMSF, 2 μ M leupeptin, 2 μ M aprotinin). After centrifugation for 1 hr at 100,000 x g, the supernatants were collected and Na₃VO₄ added to 0.1% (w/v). Immunoprecipitation and SAPK assay were as described in the Table 2 legend.

Isolated clones

p54 protein kinase was isolated from rat liver that had been stimulated with cycloheximide, using the above methods (also described in Kyriakis and Avruch, 5 *supra*; hereby incorporated by reference) and amino acid sequences were derived from peptides generated by tryptic digests. These sequences aligned with the consensus Ser/Thr protein kinase catalytic domain of known MAPKs (Hanks et al.). Two of these peptide sequences were used 10 to design overlapping degenerate oligonucleotide probes for use in a nested PCR reaction from which a 467 bp cDNA was generated using rat brain cDNA as the template. This probe was used to screen a rat brain cDNA library from which 27 clones were isolated and sequenced. The cDNAs 15 encoded five separate polypeptides (Fig. 1, SEQ ID NOS 1-5). This was quite unexpected, since it was assumed that only one peptide had been purified to homogeneity from the rat liver in this and previous work (Kyriakis and Avruch). One set of clones (p54 α I, SEQ ID NO. 9) encoded 20 a protein that contained all of the tryptic peptides derived from rat liver p54 kinase. Two additional sets (p54 β I/II, SEQ ID NOS 11 and 12; and p54 γ , SEQ ID NO. 13) encoded closely related polypeptides (88-90% identity, respectively to α I, Fig. 1). A fourth group of cDNAs 25 (p54 α II, SEQ ID NO. 10) was identical to α I kinase except for a region of 71 base pairs which results in the substitution of 15 amino acids in subdomains IX and X (see Fig. 1). The α I and α II RNAs are most likely derived from the same gene by alternate splicing. The 30 predicted proteins encoded by the full length clones are: α I, 48,076 Da; α II, 47,986 Da; and β I, 48,095 Da. Northern blotting analysis of several tissues revealed ubiquitous, low expression of all three gene classes.

Properties of p54 SAPKs

The distinct characteristics of the isolated p54 clones and expressed and native polypeptides have led to the nomenclature of Stress-Activated Protein Kinases (SAPKs), which will be used throughout to distinguish them from the MAPKs.

Sequence alignment of the catalytic domains of the p54 SAPK clones with those of the known mammalian MAPKs, and with the yeast MAPK homologs *KSS1*, *HOG-1*, *FUS3*, *SLT-2*, *spk-1* and *erk-1* (Courchesne et al., (1989), *Cell*, 58:1107-1119; Brewster et al., (1993), *Science*, 259:1760-1763; Levin et al., (1993), *J. NIH Res.*, 5:49-52) shows that the p54 polypeptides exhibit nearly equal identity to the mammalian 44 kDa MAPK (43-44% sequence identity) and the kinases from lower eukaryotes (41-44% identity). By contrast, p44 MAPK is closer in sequence to the yeast kinases (between 49-52% for the *S. cerevisiae* enzymes and 56% identity for Spk-1) than it is to the p54 SAPKs.

From these and other comparative data, we conclude that none of the MAP kinase-like genes identified thus far in lower eukaryotes is likely to be a functional homologue of the p54 SAPKs. All of the p54 isoforms contain the sequence Thr₁₈₃-Pro-Tyr₁₈₅ in an analogous position to the Thr and Tyr residues of p42/p44 MAPKs (Payne et al., (1991) *EMBO J.* 10:885-892) that must be phosphorylated for activity. These residues in the p42/p44 MAPKs are phosphorylated by the MAPK or ERK Kinases (MEKs), a family of dual specificity protein Tyr/Thr kinases (Ahn et al.; Gómez et al., (1991) *Nature*, 253:170-173; Nakielny et al., (1992), *EMBO J.*, 11:2123-2129). However, in vitro, neither purified dephosphorylated liver p54 SAPK nor bacterially expressed p54 SAPK isoforms are phosphorylated or reactivated by p42/p44 MAPK-specific MEKs, suggesting the existence of a specific p54 SAPK kinase.

In vivo administration of cycloheximide activates p54 SAPK, and we have also characterized the stimuli and signal transduction pathways that activate p54 SAPKs in cultured cells. A polyclonal antiserum (5/13-99), raised against the prokaryotic recombinant p54 SAPK β -isoform, immunoblots p54 SAPK and immunoprecipitates in vitro translated p54 α and p54 β polypeptides but not p44 MAP kinase (Fig. 2). Purified rat liver p54 SAPK phosphorylates c-Jun exclusively at Ser₇₃ and Ser₆₃ in the c-Jun trans activation domain, two sites located on c-Jun tryptic phosphopeptides designated X and Y, respectively (Pulverer et al.; Smeal et al., (1991), *Nature*, 354:494-496). The anti-p54 SAPK antiserum (5/13-99) immunoprecipitates from HT-29 human colon carcinoma cells a protein kinase activity that phosphorylates recombinant GST-c-Jun, as well as full-length c-Jun, selectively at tryptic peptides X and Y. Pretreatment of NIH3T3 cells or HT-29 cells with cycloheximide substantially augments this c-Jun (X/Y) kinase activity. Thus, the p54 antiserum is reactive with p54 SAPKs but not p42 MAPK or p44 MAPK. Moreover, p54 SAPK activity, measured as a c-Jun "X/Y" kinase, is stimulated by cycloheximide in tissue culture as well as in vivo.

SAPKs are the major c-Jun kinase activated by TNF- α . SAPK immunodepletion experiments removed 60-70% of the recombinant c-Jun kinase activity induced in TNF- α treated cells (Fig. 3). This indicates that the SAPKs account for around 70% of the c-Jun-associated c-Jun kinase activity.

30 Regulation of p54 SAPKs

Effects of MAPK Activators

We examined the regulation of the p54 SAPK, in NIH3T3 and human HT-29 cells, by agents known to regulate p42/p44 MAPKs. The activity of p54 SAPKs was measured in

an immune complex kinase assay using GST-c-Jun as a substrate, whereas the p42/p44 MAPKs in the same extracts were assayed, after Mono-Q anion exchange chromatography, using MBP as a substrate. Figure 4 compares the

5 activation of each set of kinases in response to the various treatments to the baseline values. As expected, NIH3T3 cell p42/44 MAPKs are strongly activated by mitogen (10-fold activation by FGF) and phorbol esters (6-fold), and are activated to a lesser extent by Ca^{2+}

10 influx stimulated by the ionophore A23187 (2-fold). In addition, the p42/p44 MAPKs are activated by H_2O_2 and cycloheximide (4- and 6-fold, respectively). In striking contrast, the NIH3T3 cell p54 SAPKs are not activated by FGF, phorbol esters, H_2O_2 or by Ca^{2+} ionophore (top panel,

15 filled bars). A similar result is seen for HT-29 cells (bottom panel, filled bars). In these cells, EGF substantially stimulates the p42/p44 MAPKs (6 fold, bottom panel, open bars) while only slightly stimulating p54 SAPKs (bottom panel, filled bars).

20 These results suggest that signals generated by activation of receptor tyrosine kinases and phospholipase C are not likely to represent the primary regulatory input to the p54 SAPKs. Therefore, stimuli other than mitogens were investigated to see if they could activate

25 p54 SAPKs more vigorously than they activated the p42/p44 MAPKs. Cycloheximide, although capable of strongly activating the NIH3T3 cell p54 SAPK (10-fold), also gives substantial (5-fold) activation of endogenous p42/p44 MAPKs. By contrast, heat shock (42°C , 30 min) gives a

30 large induction of NIH3T3 cell p54 SAPK activity (7-fold), while p42/p44 MAPK activity is only slightly (1.5-fold) stimulated (Fig. 4, top panel). In HT-29 cells as well, heat shock dramatically stimulates HT-29 cell p54 SAPKs (8.4 fold, Fig. 4, bottom panel, filled bars) while

35 only modestly stimulating p42/p44 MAPKs (2.9 fold, Fig.

4, bottom, open bars). Activation of p54 SAPK by heat shock (42°C) is evident by 10 min, maximal at 30 min, and declines slightly after 1 hr.

Effect of p54 SAPK Activation on c-Jun

5 Since the p54 SAPKs are potent c-Jun kinases *in vitro*, we inquired whether stimuli such as heat shock and cycloheximide, that activate p54 SAPKs preferentially, also increase the phosphorylation of c-Jun *in situ*. ³²P-labeled U937 and HepG2 cells were exposed to heat shock
10 or cycloheximide, and the phosphorylation of endogenous c-Jun was examined. Both treatments induce a robust phosphorylation of c-Jun, accompanied by a dramatic retardation of c-Jun mobility on SDS-PAGE (Fig. 5a). This retardation is characteristic of phosphorylation on
15 tryptic peptides X and Y (corresponding to Ser 73 and 63, respectively) within the c-Jun N-terminal trans activation domain (Pulverer et al.); the induction of X/Y phosphorylation was verified directly by tryptic peptide maps of ³²P-c-Jun isolated from heat-shocked ³²P-labeled
20 HepG2 cells (Fig. 5b). Activation of c-Jun N-terminal phosphorylation by heat shock represents a previously unrecognized mode of c-Jun regulation. The likelihood that p54 SAPKs contribute substantially to the c-Jun phosphorylation elicited by heat shock and cycloheximide
25 *in situ* is supported by the ability of the immunoprecipitated p54 SAPK activated by cycloheximide to phosphorylate recombinant c-Jun *in vitro* exclusively on tryptic ³²P peptides that co-migrate with tryptic peptides X and Y (see Fig. 3).

30 *Effects of Protein Synthesis Inhibitors on p54 SAPKs*

Maneuvers that increase c-Jun X/Y phosphorylation *in situ*, such as UV light or PMA, have been shown to activate c-Jun kinases that bind tightly to immobilized

GST-Jun (Adler et al.; Hibi et al., *Genes & Dev.*, 7:2135-2148, 1993). Extracts from heat-shocked or cycloheximide-treated cells also contain activated c-Jun kinase(s) that bind to immobilized GST-c-Jun (Fig. 6, right panel). Moreover, two other inhibitors of polypeptide chain elongation, emetine and especially anisomycin, also activate both p54 SAPK and GST-Jun-bound c-Jun kinase (Fig. 6), whereas puromycin and the RNA synthesis inhibitor, actinomycin D, are each unable to activate either c-Jun kinase (Fig. 6, right panel) or p54 SAPK activity (Fig. 6, left panel). A summary of these results is presented in Table 1, below. Thus, heat shock, a variety of protein synthesis inhibitors as well as the glycosylation inhibitor, tunicamycin (see below), alter p54 SAPK, and the GST-Jun-bound c-Jun kinase in parallel, suggesting that p54 SAPK is likely to be one of the physiologic Jun kinases activated by this class of perturbations.

TABLE 1

Activation of p54s and c-Jun-associated kinases by protein and RNA synthesis inhibitors.

| <u>Treatment</u> | <u>p54 MAPK (mU)</u> | <u>c-Jun-associated kinase (mU)</u> |
|------------------|----------------------|-------------------------------------|
| Control | 31.0 | 14.5 |
| 25 Anisomycin | 370.0 | 385.0 |
| Cycloheximide | 166.0 | 243.0 |
| Heat-shock | 168.0 | 180.0 |
| Emetine | 75.0 | 85.0 |
| Puromycin | 19.3 | 21.5 |
| 30 Actinomycin-D | 22.0 | 24.0 |

Activation of p54s and c-Jun-associated kinases by protein and RNA synthesis inhibitors. U937 cells were treated with anisomycin (10µg/ml, 30 min), cycloheximide

(200 μ M, 60 min), heat shock (42°C, 30 min), or actinomycin D (10 μ g/ml, 30 min). Extracts were prepared as described in the methods, and parallel aliquots were subjected to GST-c-Jun chromatography/assay or immunoprecipitation and assay for p54. In both assays, 1 unit of kinase activity transferred 1 pmol PO₄/min from ATP to GST-c-Jun. Shown are mean results for two experiments.

Polypeptide Misfolding Stimulates p54 SAPKs

- 10 Although heat shock and protein synthesis inhibitors could activate p54 SAPK through entirely unrelated mechanisms, a shared property of heat shock and translational inhibitors (but not the RNA synthesis inhibitor, actinomycin D), is their ability to promote
- 15 polypeptide misfolding and denaturation. Consequently, we investigated whether the accumulation of misfolded polypeptides might be an initial stimulus common to protein synthesis inhibitors and heat shock that leads to the preferential activation of p54 SAPK. Tunicamycin
- 20 inhibits the synthesis and proper folding of proteins destined for membrane insertion or secretion through inhibition of N-linked glycosylation in the Golgi. Increasing doses of tunicamycin added to HT29 cells promotes a striking activation (up to 12-fold) of p54
- 25 SAPK activity (Fig. 7, top, filled circles), whereas the p42/p44 MAPKs, assayed after separation by Mono-Q chromatography, are only modestly activated (Fig. 7, bottom). In addition, tunicamycin stimulates total HT-29 Jun kinases which bind to GST-c-Jun with a similar dose
- 30 response to p54 SAPK activation (Fig. 7, top, open circles). The activation of the p54 SAPKs by tunicamycin, together with the data in Figures 4 and 6 support the idea that cellular stresses which result in the accumulation of misfolded polypeptides, can, to a
- 35 degree greater than mitogens, generate a signal to activate the p54 SAPKs. By contrast, the p42/p44 MAPKs

are more strongly activated by mitogenic signaling,
relative to stress signaling.

Cytokines TNF- α and IL-1- β

In mammals, integration of the multicellular and
5 inter-organ response to a variety of "stressful" noxious
stimuli is mediated by a diverse array of inflammatory
cytokines, such as TNF- α and IL-1- β . TNF- α was initially
detected by its ability to induce the hemorrhagic
necrosis of some transplantable tumors in inbred mice
10 (Buetler et al., *Ann. Rev. Biochem.*, 57:505-517, 1988;
Goeddel et al., *Cold Spring Harbor Symp. Quant. Biol.*,
51:597-609, 1986). The cellular responses to
inflammatory cytokines are quite diverse and cell-
specific, and are directed at optimizing overall host
15 defense against infection. For example, TNF- α acts on
adipose tissue to inhibit insulin action and energy
storage; on liver to yield the protein secretory pattern
known as the acute phase response; on neutrophils to
enhance superoxide radical production and cytotoxic
20 efficacy; and on T cells to promote the secretion of
additional cytokines, e.g. IL-2 and IL-6 (see Buetler et
al. and Goeddel et al. for review). The multifarious
actions of TNF- α are due in part to TNF- α -directed
programs of gene expression. Notably, TNF- α has been
25 shown to be a potent activator of the trans activation
function and autoinduction of c-Jun (Brenner et al.,
Nature, 337:661-663, 1989), as well as an activator of
NF- κ B (Osborn et al., *Proc. Natl. Acad. Sci. U.S.A.*,
86:2336-2340, 1989), both of which are needed by
30 lymphocytes for trans activation of the IL-2 gene.

The profile of physiologic and cellular responses
to TNF- α and IL-1- β led us to inquire whether these
agents could activate p54 SAPK. Human CCD-18Co colon
fibroblasts are acutely responsive to TNF- α (Goeddel et

al.); in these cells, TNF- α elicited a striking activation of p54 SAPKs, whereas EGF and PMA were without noteworthy effect (Fig. 8a). The 10-fold stimulation of p54 SAPK by TNF- α was slightly greater than that provoked by heat stress. Comparing the responses of p42/p44 MAPKs in CCD-18Co cells, it is clear that heat stress and TNF- α are far more potent activators of p54 SAPK than are EGF and PMA, while the converse is true for the p42/44 MAPKs (Fig. 8b).

10 Liver is also a target tissue for TNF- α (Buetler et al., and Goeddel et al.); addition of TNF- α to primary cultures of freshly isolated porcine hepatocytes stimulates p54 SAPK activity by \approx 6-fold (Fig. 9a), whereas EGF increases p54 SAPK activity 3-fold. In these
15 cells, EGF and PMA activate the p42/p44 MAPKs and the p54 SAPKs to a comparable degree. However, as is seen in CCD-18Co cells, heat shock and TNF- α are much more potent activators of p54 SAPK than they are p42/p44 MAPK activators (Fig. 9b). The present results are thus
20 consistent with recent reports showing that TNF- α activates p42/p44 MAPKs (Van Lint et al., *J. Biol. Chem.*, 267:25916-25921, 1992); however, it is clear that the p42/p44 MAP kinases are much more potently activated by ligands like EGF and FGF, that operate through receptor
25 tyrosine kinase, whereas the ability of TNF- α to activate the p54 SAPKs greatly exceeds the ability of FGF and EGF to activate the p54 SAPK, at least in most cell types.

Little is known of the molecular mechanisms of TNF- α signaling. TNF- α binds to one of two receptors,
30 55-kDa and 70-kDa, whose intracellular extensions show no homology with receptors whose signal transduction mechanisms are better understood (Loetscher et al., *Cell*, 61:351-359, 1990; Heller et al., *Proc. Natl. Acad. Sci. U.S.A.*, 87:6151-6155, 1990). TNF- α has been shown to
35 stimulate rapid sphingomyelin hydrolysis and the

accumulation of ceramide, through the activation of a neutral sphingomyelinase (Dressler et al.; Yang et al., *J. Biol. Chem.*, 268:20520-20523, 1993; Dobrowsky et al., *J. Biol. Chem.*, 267:5048-5051, 1992; Dbaiibo et al., *J. Biol. Chem.*, 268:17762-17766, 1993; Schütze et al., *Cell*, 71:42-52, 1992). Ceramide has been proposed to serve as a second messenger for TNF- α , analogous to the role envisioned for diacylglycerol in the action of hormones that activate phosphatidylinositol-specific phospholipase C enzymes (Dressler, et al.; Yang, Z., et al.; Dobrowsky, et al.; Dbaiibo, et al.; Schütze et al.; and Schütze, et al.). Many of the responses to TNF- α , including growth inhibition, apoptosis, activation of heterotrimeric forms of protein phosphatase-2A, activation of a membrane bound Ser/Thr kinase and, possibly, activation of NF- κ B can be elicited by the addition of various ceramide derivatives or bacterial sphingomyelinase to intact or permeabilized cells (Dressler et al.; Yang, et al.; Dobrowsky, et al.; Dbaiibo, et al.; and Schütze et al.). Based on these considerations, we compared the ability of TNF- α , IL-1- β and *S. aureus* sphingomyelinase treatment of HepG2 or EL-4 cells to alter p54 SAPK activity. TNF- α stimulates a robust activation of the p54 SAPKs (15-fold) in these cells, and a substantial activation of p54 SAPKs (5.3-fold) is also evident in response to sphingomyelinase (Fig. 10). These results are consistent with the possibility that sphingomyelin hydrolysis, as is known to occur in response to inflammatory cytokines, may be an early step in the p54 SAPK signal transduction pathway.

When EL-4 murine thymoma cells were treated with 20 ng/ml recombinant human IL-1- β , p54 SAPK activity increased over 4-fold, while p42/44 MAPK activity remained unchanged by the treatment (Fig. 11 a and b). This strong induction of SAPK activity without

concomitant activation of MAPKs mirrors the results with cytokine TNF- α , and indicates a similar inflammation-mediated pathway.

Ischemia/Reperfusion Induces p54 SAPKs

5 An additional distinction for the p54 SAPKs from the p42/44 MAPKs is the time course of activation in response to reperfusion of ischemic tissue. Rat kidney was made ischemic *in vivo* by the methods described above. The results of this experiment (Fig. 12) showed that the
10 p54 SAPKs and the p42/44 MAPKs both were activated immediately upon reperfusion of the kidney, and that the activation of p54 SAPKs peaked after about 20 min (Fig. 12a), while the p42/44 MAPKs peaked at about 5 min after initiation of reperfusion (Fig. 12b). The duration of
15 p54 SAPK activation was also considerably longer, lasting considerably longer than 90 min before returning to control levels, whereas p42/44 MAPK activation returned to control levels within 20 min.

These findings have important implications in the
20 pathogenesis of chronic problems such as acute renal failure, infarction arising from ischemic heart disease, and surgical induction of ischemia/reperfusion injury. The p54 SAPKs clearly play a more significant role in tissue stresses such as ischemia than do the p42/44
25 MAPKs, and their effects are more sustained. These results are the first to delineate a signal transduction cascade specifically activated during reperfusion of ischemic tissue. It is known that c-fos and some heat shock protein genes are transcribed during reperfusion,
30 and that ischemia results in the generation of oxygen radicals and can lead to tissue damage. The data presented here indicate that SAPKs are activated during reperfusion, and SAPKs target c-Jun. Tissue repair genes, such as the collagenase gene, are regulated by AP-

1, and thus, SAPK activation may represent the initiation of repair processes at the molecular level.

Therapeutic Drug Screening with p54 SAPKs

The experimental approaches described above can be used to screen compounds to identify those with therapeutic potential. Two examples of possible screening protocols follow. These examples are not intended to be limiting.

Example 1

Human (or other mammalian) cell lines such as HepG2, CCD-18Co, U937, and HT-29 can be treated with any test compound, and treated with SAPK activators (e.g., TNF- α , IL-1- β , ATP-depletion/refeeding analogous to ischemia/reperfusion, etc.). The order of treatment can be test compound, then SAPK-activator; SAPK activator then test compound, or both treatments simultaneously. Treatment times can vary from 1 min to several hours, depending on the time necessary to induce changes in the SAPK pathway.

The cells are then extracted, and the endogenous SAPKs are immunoprecipitated as described above. The SAPKs are then tested in a standard c-Jun kinase assay for activity. This method of assaying for SAPK activation can also be employed with tissue samples obtained from patient biopsy, and has been used successfully to detect the activity of SAPKs from samples of rat kidney. In this way, therapeutics can be evaluated for their ability to activate or inhibit the SAPK pathway.

Example 2

Any of the human or rat SAPK clones may be used in an expression vector for recombinant bacterial (or viral, etc.) expression of inactive forms of the SAPK. Because of the high homology between rat and human SAPKs, either

may be used in the assay for therapeutic compounds. Human cell lines such as HepG2, CCD-18Co, U937, and HT-29 can be treated with any compound to evaluate its toxicity and efficacy as a therapeutic in modulating the SAPK/c-Jun kinase pathway.

Cells are treated with the test compound either alone or with a SAPK activating compound (such as TNF- α , etc.; the activator may be applied before or concurrently with the application of the test compound), and then extracted to produce a cytoplasmic lysate. This extract is then combined with the recombinantly expressed, inactive SAPK. The SAPK can be expressed as a GST fusion protein so that it is easily purified on glutathione agarose. After a suitable length of time (minutes to hours) for interaction between the cell lysate and the recombinant inactive SAPK, the recombinant SAPK is isolated by binding to glutathione beads (leaving behind endogenous SAPKs) or by immunoprecipitation with an anti-glutathione antibody (commercially available; any other isolation technique may be used also), and assayed for c-Jun kinase activity. If there is no c-Jun kinase activity, and the test compound was applied to the cells alone, then it may be concluded that this compound does not activate SAPKs. If the test compound was applied with a SAPK activator, then it may be concluded that the test compound inhibits the activation of the SAPK pathway. If c-Jun kinase activity is detected, and the test compound was applied to the cells alone, then the compound activates the SAPK pathway. If the test compound was applied with a SAPK activator, and c-Jun kinase activity is detected, then it may be concluded that a) the compound does not inhibit the SAPK pathway, b) the compound may enhance SAPK activity (which can be quantitatively evaluated), or c) the drug has no effect.

Both of these screening systems allow rapid and extensive testing of many compounds, regardless of their preconceived potential therapeutic use, and may facilitate identification of therapeutics which accelerate tissue repair (increased SAPK pathway activation), or prevent or alleviate allergy, excess swelling, anaphylaxis, etc. (decreased SAPK pathway activation). An example of this assay is shown in Figure 13, using TNF- α as the test compound, and showing controls with the expressed SAPK alone, and the H ϵ pG2 cell extract alone to demonstrate that the recombinant form is inactive as expressed, and that the cell extract has no intrinsic activity in the assay.

Summary of Results

This invention identifies the molecular structures of a new subfamily of proline-directed protein kinases whose upstream activators include heat stress, protein synthesis inhibitors, ischemia/reperfusion injury, and the inflammatory cytokines, TNF- α and IL-1- β . This array of regulatory inputs suggests that this kinase subfamily may serve to monitor and form part of the cellular response to a variety of intracellular and extracellular stress signals. The p42/p44 MAPKs, although capable of being activated by these stress treatments, are to a much greater degree activated by mitogens (see Table 2 for summary of results). The mitogenic agents such as EGF, FGF and PMA, which have been shown to activate the p42/p44 MAPKs through a Ras and c-Raf-1-dependent pathway, activate the SAPKs weakly if at all. This suggests that the SAPKs, although requiring both Ser/Thr and Tyr phosphorylation for activity (Kyriakis et al., (1991), *J. Biol. Chem.*, 266:10043-10046) as do the p42/p44 MAPKs (Anderson et al.), lie on an entirely distinct signal transduction pathway. Consistent with

this conclusion is our inability, using highly purified MEK, to reactivate phosphatase-2A inactivated rat liver SAPK, or to activate prokaryotic recombinant SAPK- β under conditions that provide near complete MEK activation of comparable preparations of purified or recombinant p42/p44 MAPK. The SAPKs are thus the proline-directed kinase elements in what is likely to be a new protein kinase cascade, the second such pathway uncovered in mammalian cells. Three independently regulated protein kinase cascades upstream of distinct proline-directed kinases (*FUS/KSS*; *HOG-1*; *MPK-1*) have been uncovered thus far in *S. cerevisiae* (Levin et al.). We propose that the SAPK cascade is an important, perhaps central, component of the cellular response system to TNF- α , analogous to the role of the MAPK cascade in the response to activation of receptor tyrosine kinases. Lipid regulators derived from sphingomyelin hydrolysis may participate at one of the earlier intracellular steps upstream of the SAPKs; a similar role for phospholipid-derived mediators upstream of the MAPKs has recently been proposed (Cai et al., (1993), *Mol. Cell. Biol.*, 13:7645-7651).

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TABLE 2

Activation of p54 and MAPKs P42/44 In various cell lines by various treatments. ND = not done.

| 5 | Cells Activity | Treatment | p54 Activity (mU) | p42/44 MAPK |
|----|-----------------------------------|-------------------------------|----------------------|----------------|
| | NIH3T3 | Control | 5.8 ± 0.6 | 71.4 |
| | | PMA | 5.8 ± 0.9 | 409.7 |
| | | FGF | 8.5 ± 0.6 | 795.5 |
| 10 | | A23187 | 8.9 ± 0.7 | 172.0 |
| | | H ₂ O ₂ | 5.9 ± 0.1 | 272.2 |
| | | Heat shock | 38.8 ± 1.7 | 169.7 |
| | | Cycloheximide | 57.1 ± 4.1 | 419.9 |
| | HT-29 | Control | 20.7 ± 1.7 | 95.0 |
| 15 | | EGF | 35.7 ± 1.4 | 576.0 |
| | | Heat shock | 172.0 ± 28.5 | 281.0 |
| | HT-29 | Control | 69.4 ± 7.5 | 195.5 |
| | | Tunicamycin | 803.9 ± 77.1 | 480.9 |
| | CCD-18Co | Control | 7.0 ± 1.0 | 12.2 |
| 20 | | PMA | 8.8 ± 0.7 | 601.0 |
| | | EGF | 13.0 ± 2.1 | 394.0 |
| | | Heat shock | 48.0 ± 1.3 | 83.7 |
| | | TNF-α | 60.0 ± 5.0 | 117.6 |
| 25 | Primary Porcine Hepatocytes | Control | 179 ± 13 | 1294 |
| | | PMA | 479 ± 58 | 3529 |
| | | EGF | 665 ± 58 | 4882 |
| | | Heat shock | 697 ± 49 | 2427 |
| | | TNF-α, 10 min | 1008 ± 31 | 3064 |
| 30 | | TNF-α, 20 min | 924 ± 38 | ND |
| | HepG2 | Control | 29.8 ± 4.3 | ND |
| | | TNF-α | 434.8 ± 36.4 | ND |
| | | Sphingomyelinase | 159.0 ± 17.3 | ND |

Confluent cultures of NIH3T3 cells were
 35 treated with H₂O₂ (5 mM, 15 min) phorbol-12-myristate-13-
 acetate (PMA, 500 nM, 20 min) FGF (10 ng ml⁻¹, 20 min),
 A23187 (100 nM 20 min), heat (42°C, 30 min) or
 cycloheximide (200 μM, 60 min). HT-29 cells were treated
 with EGF (50 ng ml⁻¹, 20 min), heat shock (42°C, 30 min)
 40 or tunicamycin (50 μ ml⁻¹). CCD-18Co Cells (100%
 confluent) were treated with PMA 200 nM, 15 min), EGF (50
 ng ml⁻¹, 15 min), heat shock (42°C, 40 min) or TNF-α (50
 ng ml⁻¹, 20 min). Primary porcine hepatocytes were
 treated the same as the CCD-18Co cells. HepG2 cells (80%
 45 confluent) were treated with TNF-α (50 ng ml⁻¹, 15 min) or

S. aureus sphingomyelinase (100 U ml⁻¹). 1U *S. aureus* sphingomyelinase will hydrolyze 1 μ mole TNP-sphingomyelin min⁻¹ at pH 7.4, 37°C. Cell lysis and immunoprecipitation were performed as described in Kyriakis et al, *Nature* 358:417-421, 1992 (hereby incorporated by reference).
 5 Precipitates were assayed for GST-Jun kinase activity as follows. To 40 μ l of a 1:1 suspension of protein G Sepharose beads containing immunocomplexed p54 were added 20 μ l 0.2 mg ml⁻¹ GST-C-Jun-1-135 (26,37) or 0.01 mg ml⁻¹
 10 holo-c-Jun. [γ -³²P]ATP (100 μ M) and MgCl₂ (10 mM) were added to start the reaction. The reaction was allowed to proceed for 20 min at 30°C at which time the reaction was stopped with SDS sample buffer and the mixtures resolved by SDS-PAGE. The 40-kDa GST-Jun band was excised and
 15 counted by liquid scintillation spectrometry. p54 kinase assays were performed in triplicate. Mean \pm SD are shown. Another portion of extracts (1 ml) was subjected to Mono-Q chromatography; fractions were assayed for MAPK p42/44 activity as MBP kinase activity. A peak of
 20 stimulated MBP kinase activity was always detected eluting between 200 and 350 mM NaCl; total MAPK p42/44 activity was taken as MBP kinase activity in those fractions combined. 1 U p54 activity will transfer 1 pmol min⁻¹ PO₄ from ATP to c-Jun. 1 U MAPK p42/44 will
 25 transfer 1 pmol min⁻¹ PO₄ from ATP to MBP.

A central conundrum in the field of signal transduction is how agonists such as EGF and TNF- α can exert such different effects while apparently only activating one MAPK (the p42/p44 MAPK) pathway. The data
 30 in Figures 16 and 17, taken in combination with the known differences in SAPK and p42/p44 MAPK substrate specificity, suggest that signaling specificity may arise in part from the differential recruitment of signaling pathways such as the SAPK and p42/p44 MAPK pathways.

35 Whatever the identity of the elements that couple heat shock, protein synthesis inhibition, the cytokine receptor(s), and other cellular stresses to the SAPKs, the results presented here support the contention that the c-Jun polypeptide is a downstream target of the
 40 SAPKs. The SAPKs are a group of c-Jun N-terminal kinases. These kinases and members of the related superfamily focus a broad variety of regulatory signals, including phorbol esters, Ha-Ras, ultraviolet radiation

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(Pulverer et al.; Smeal et al.; Adler et al.; Hibi et al.; Binétruy et al.; Devary et al., (1992), Cell, 71:1081-1091), heat shock, protein synthesis inhibitors and cytokines into the phosphorylation of the c-Jun trans
5 activation domain. The multiplicity of these c-Jun kinases and their activating inputs attest to the diversity of c-Jun function, and the complexity of its regulation. The role of c-Jun phosphorylation in the heat shock response and in the actions of TNF- α remain to
10 be clarified. The consequences of c-Jun mediated transcriptional trans activation exhibit a great deal of cell specificity, and whether the outcome of MAPK- or SAPK-mediated c-Jun transactivation is mitogenesis, growth inhibition or a stable new phenotype resulting
15 from altered gene expression is likely to depend on the array of signaling components acting in concert with AP-1.

Uses of the Invention

The extracellular signal regulated family of
20 kinases are activated in response to different extracellular stimuli, and this specificity allows cells to diverge in their function in order to target a response to a given stimulus. The molecules of the invention can intervene in or stimulate a basic pathway
25 modulating transcription of proteins that mediate an inflammatory or cell-stress response. Their use in the treatment and prevention of inflammation and the deleterious effects of hypoxia, heat stress, reperfusion injury, and other tissue insults may resolve many
30 difficulties that arise with current therapies that rely on amelioration of the aftermath of damage instead of being able to redirect the course of the syndrome.

An additional use for these molecules may be in the upregulation of IL-2, which has proven useful in the

treatment of cancer. AP-1 activation is known to induce IL-2, and since c-Jun is a component of the AP-1 dimer, p54 phosphorylation of c-Jun would be expected to modulate AP-1 levels. p54 immunodepletion experiments have been shown to have a major effect on the phosphorylation of c-Jun.

A use for the p54 kinases is as a template for drug design, or a functional component for therapeutic drug assays. Many functional motifs of the molecules are already known (e.g., the ATP-binding site, sites of regulatory phosphorylation, c-Jun binding site, etc.) which, if an effective antagonist or agonist could be derived, could play an important role in therapies for conditions such as are listed above. Assays to screen for such drugs exist, and are described above. Monitoring the enzymatic activity following incubation with potentially therapeutic compounds such as was done with the drugs described herein would allow a simple, fast method to determine efficacy and dose-response for up or down regulation of the SAPKs. Large numbers of candidate compounds can be screened easily and evaluated for specificity, efficacy, and toxicity. Such information would allow rational evaluation of many drugs for their ability to up- or down-regulate cellular responses to physiological stresses, and be useful in clinical management of inflammation, ischemia, and many other stimuli that activate the SAPKs.

The molecules of the invention may be useful for reducing inflammation in such chronic disorders as autoimmune diseases or allergies, and in acute conditions such as anaphylactic shock, or soft tissue injury where swelling may aggravate the condition (e.g, around broken bones). Other uses include prophylactic treatment of patients about to undergo surgeries where there is a high likelihood of ischemia-reperfusion injury (e.g., vascular

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surgery, organ transplants, etc.), or treatment of sepsis and fever.

For such conditions as mentioned above, a systemic application of the molecules of the invention, 5 or antibodies derived from them, is generally desirable, although local application may be more appropriate in certain cases. Transport of the molecules to their site of action may be effected by, for example, liposome delivery systems, antisense technology, plasmid or 10 retroviral vectors, or any of a number of other methods known in the art. The vehicle for application may be any excipient compatible with the molecules and the health of the patient.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: KYRIAKIS, JOHN M.
AVRUCH, JOSEPH
BANERJEE, PAPIA
WOODGETT, JAMES R.
- (ii) TITLE OF INVENTION: p54 STRESS-ACTIVATED PROTEIN
KINASES
- (iii) NUMBER OF SEQUENCES: 16
- (iv) CORRESPONDENCE ADDRESS:
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 - (E) COUNTRY: US
 - (F) ZIP: 02110
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
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- (viii) ATTORNEY/AGENT INFORMATION:
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 - (C) REFERENCE/DOCKET NUMBER: 00786/217001
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 - (C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 423 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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 20           25           30
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu
 35           40           45
Gly Ile Asn Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
 50           55           60
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val
 65           70           75           80
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
 85           90           95
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp
100           105           110
Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met
115           120           125
Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
130           135           140
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145           150           155           160
Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165           170           175
Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180           185           190
Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile
195           200           205
Trp Ser Val Gly Cys Ile Met Ala Glu Met Val Leu His Lys Ser Cys
210           215           220
Ser Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225           230           235           240
Leu Gly Thr Pro Ser Ala Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245           250           255
Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Pro Gly Ile Lys Phe Glu
260           265           270
Glu Leu Phe Pro Asp Trp Ile Phe Pro Ser Glu Ser Glu Arg Asp Lys
275           280           285
Ile Lys Thr Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290           295           300

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Asp Pro Asp Lys Arg Ile Ser Val Asp Glu Ala Leu Arg His Pro Tyr
305 310 315 320
Ile Thr Val Trp Tyr Asp Pro Ala Glu Ala Glu Ala Pro Pro Pro Gln
325 330 335
Ile Tyr Asp Ala Gln Leu Glu Glu Arg Glu His Ala Ile Glu Glu Trp
340 345 350
Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Trp Glu Glu Arg Ser Lys
355 360 365
Asn Gly Val Lys Asp Gln Pro Ser Asp Ala Ala Val Ser Ser Lys Ala
370 375 380
Thr Pro Ser Gln Ser Ser Ser Ile Asn Asp Ile Ser Ser Met Ser Thr
385 390 395 400
Glu His Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Asp Ala Ser Thr
405 410 415
Gly Pro Leu Glu Gly Cys Arg
420

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 423 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ser Asp Ser Lys Ser Asp Gly Gln Phe Tyr Ser Val Gln Val Ala
1 5 10 15
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Gln Leu Lys Pro Ile
20 25 30
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Phe Asp Thr Val Leu
35 40 45
Gly Ile Asn Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Leu Lys Cys Val
65 70 75 80
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp
100 105 110
Ala Asn Leu Cys Gln Val Ile His Met Glu Leu Asp His Glu Arg Met
115 120 125
Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
130 135 140
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160

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Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175

Cys Thr Asn Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190

Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile
195 200 205

Trp Ser Val Gly Cys Ile Met Gly Glu Leu Val Lys Gly Cys Val Ile
210 215 220

Phe Gln Gly Thr Asp His Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225 230 235 240

Leu Gly Thr Pro Ser Ala Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245 250 255

Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Pro Gly Ile Lys Phe Glu
260 265 270

Glu Leu Phe Pro Asp Trp Ile Phe Pro Ser Glu Ser Glu Arg Asp Lys
275 280 285

Ile Lys Thr Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290 295 300

Asp Pro Asp Lys Arg Ile Ser Val Asp Glu Ala Leu Arg His Pro Tyr
305 310 315 320

Ile Thr Val Trp Tyr Asp Pro Ala Glu Ala Glu Ala Pro Pro Pro Gln
325 330 335

Ile Tyr Asp Ala Gln Leu Glu Glu Arg Glu His Ala Ile Glu Glu Trp
340 345 350

Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Trp Glu Glu Arg Ser Lys
355 360 365

Asn Gly Val Lys Asp Gln Pro Ser Asp Ala Ala Val Ser Ser Lys Ala
370 375 380

Thr Pro Ser Gln Ser Ser Ser Ile Asn Asp Ile Ser Ser Met Ser Thr
385 390 395 400

Glu His Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Asp Ala Ser Thr
405 410 415

Gly Pro Leu Glu Gly Cys Arg
420

(2) INFORMATION FOR SEQ ID NO:3:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 426 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Ser Lys Ser Lys Val Asp Asn Gln Phe Tyr Ser Val Glu Val Gly
1 5 10 15

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Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Asn Leu Lys Pro Ile
20 25 30

Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Tyr Asp Ala Val Leu
35 40 45

Asp Arg Asn Val Ala Ile Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60

Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Met Lys Cys Val
65 70 75 80

Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95

Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp
100 105 110

Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu Asp His Glu Arg Met
115 120 125

Ser Tyr Leu Leu Tyr Gln Met Leu Ser Ala Ile Lys His Leu His Ser
130 135 140

Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160

Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175

Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190

Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile
195 200 205

Trp Ser Val Gly Cys Ile Met Gly Glu Met Val Arg His Lys Ile Leu
210 215 220

Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225 230 235 240

Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245 250 255

Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Ala Gly Leu Thr Phe Pro
260 265 270

Lys Leu Phe Pro Asp Ser Leu Phe Pro Ala Asp Ser Glu His Asn Lys
275 280 285

Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290 295 300

Asp Pro Ala Lys Arg Ile Ser Val Asp Asp Ala Leu Gln His Pro Tyr
305 310 315 320

Ile Asn Val Trp Tyr Asp Pro Ala Glu Val Glu Ala Pro Pro Pro Gln
325 330 335

Ile Tyr Asp Lys Gln Leu Asp Glu Arg Glu His Thr Ile Glu Glu Trp
340 345 350

Lys Glu Leu Ile Tyr Lys Glu Val Met Asn Ser Glu Glu Lys Thr Lys
355 360 365

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Asn Gly Val Val Lys Gly Gln Pro Ser Pro Ser Gly Ala Ala Val Asn
370 375 380
Ser Ser Glu Ser Leu Pro Pro Ser Ser Ser Val Asn Asp Ile Ser Ser
385 390 395 400
Met Ser Thr Asp Gln Thr Leu Ala Ser Asp Thr Asp Ser Ser Leu Glu
405 410 415
Ala Ser Ala Gly Pro Leu Gly Cys Cys Arg
420 425

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 385 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ser Lys Ser Lys Val Asp Asn Gln Phe Tyr Ser Val Glu Val Gly
1 5 10 15
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Asn Leu Lys Pro Ile
20 25 30
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Tyr Asp Ala Val Leu
35 40 45
Asp Arg Asn Val Ala Ile Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Met Lys Cys Val
65 70 75 80
Asn His Lys Asn Ile Ile Ser Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95
Thr Leu Glu Glu Phe Gln Asp Val Tyr Leu Val Met Glu Leu Met Asp
100 105 110
Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu Asp His Glu Arg Met
115 120 125
Ser Tyr Leu Leu Tyr Gln Met Leu Ser Ala Ile Lys His Leu His Ser
130 135 140
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160
Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175
Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190
Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Ile
195 200 205
Trp Ser Val Gly Cys Ile Met Gly Glu Met Val Arg His Lys Ile Leu
210 215 220

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Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225 230 235 240
Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245 250 255
Arg Asn Tyr Val Glu Asn Arg Pro Lys Tyr Ala Gly Leu Thr Phe Pro
260 265 270
Lys Leu Phe Pro Asp Ser Leu Phe Pro Ala Asp Ser Glu His Asn Lys
275 280 285
Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290 295 300
Asp Pro Ala Lys Arg Ile Ser Val Asp Asp Ala Leu Gln His Pro Tyr
305 310 315 320
Ile Asn Val Trp Tyr Asp Pro Ala Glu Val Glu Ala Pro Pro Pro Gln
325 330 335
Ile Tyr Asp Lys Gln Leu Asp Glu Arg Glu His Thr Ile Glu Glu Trp
340 345 350
Lys Glu Leu Ile Tyr Lys Glu Val Met Asn Ser Glu Glu Lys Thr Lys
355 360 365
Asn Gly Val Val Lys Gly Gln Pro Ser Pro Ser Xaa Xaa Gly Ala Ala
370 375 380
Val
385

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Ser Arg Ser Lys Arg Asp Asn Asn Phe Tyr Ser Val Glu Ile Ala
1 5 10 15
Asp Ser Thr Phe Thr Val Leu Lys Arg Tyr Gln Asn Leu Lys Pro Ile
20 25 30
Gly Ser Gly Ala Gln Gly Ile Val Cys Ala Ala Tyr Asp Ala Ile Leu
35 40 45
Glu Arg Asn Val Ala Ile Lys Lys Leu Ser Arg Pro Phe Gln Asn Gln
50 55 60
Thr His Ala Lys Arg Ala Tyr Arg Glu Leu Val Leu Met Lys Cys Val
65 70 75 80
Asn His Lys Asn Ile Ile Gly Leu Leu Asn Val Phe Thr Pro Gln Lys
85 90 95
Ser Leu Glu Glu Phe Gln Asp Val Tyr Ile Val Met Glu Leu Met Asp
100 105 110

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Ala Asn Leu Cys Gln Val Ile Gln Met Glu Leu Asp His Glu Arg Met
115 120 125
Ser Tyr Leu Leu Tyr Gln Met Leu Cys Gly Ile Lys His Leu His Ser
130 135 140
Ala Gly Ile Ile His Arg Asp Leu Lys Pro Ser Asn Ile Val Val Lys
145 150 155 160
Ser Asp Cys Thr Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg Thr Ala
165 170 175
Gly Thr Ser Phe Met Met Thr Pro Tyr Val Val Thr Arg Tyr Tyr Arg
180 185 190
Ala Pro Glu Val Ile Leu Gly Met Gly Tyr Lys Glu Asn Val Asp Leu
195 200 205
Trp Ser Val Gly Cys Ile Met Gly Glu Met Val Cys Leu Lys Ile Leu
210 215 220
Phe Pro Gly Arg Asp Tyr Ile Asp Gln Trp Asn Lys Val Ile Glu Gln
225 230 235 240
Leu Gly Thr Pro Cys Pro Glu Phe Met Lys Lys Leu Gln Pro Thr Val
245 250 255
Arg Thr Tyr Val Glu Asn Arg Pro Lys Tyr Ala Gly Tyr Ser Phe Glu
260 265 270
Lys Leu Phe Pro Asp Val Leu Phe Pro Ala Asp Ser Glu His Asn Lys
275 280 285
Leu Lys Ala Ser Gln Ala Arg Asp Leu Leu Ser Lys Met Leu Val Ile
290 295 300
Asp Ala Ser Lys Arg Ile Ser Val Asp Glu Ala Leu Gln His Pro Tyr
305 310 315 320
Ile Asn Val Trp Tyr Asp Pro Ser Glu Ala Glu Ala Pro Pro Pro Lys
325 330 335
Ile Pro Asp Lys Gln Leu Asp Glu Arg Glu His Thr Ile Glu Glu Trp
340 345 350
Lys Glu Leu Ile Tyr Lys Glu Val Met Asp Leu Glu Glu Arg Thr Lys
355 360 365
Asn Gly Val Ile Arg Gly Gln Pro Ser Pro Leu Gly Ala Ala Val Ile
370 375 380
Asn Gly Ser Gln His Pro Val Ser Ser Pro Ser Val Asn Asp Met Ser
385 390 395 400
Ser Met Ser Thr Asp Pro Thr Leu Ala Ser Asp
405 410

(2) INFORMATION FOR SEQ ID NO:6:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Asp Ala Ala Val Ser Ser Lys Ala Thr Pro Ser Gln Ser Ser
1 5 10 15
Ile

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Ala Ala Val Asn Ser Ser Glu Ser Leu Pro Pro Ser Ser Ser Val
1 5 10 15
Gln Pro Ser Pro Ser
20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser Pro Leu Gly Ala Ala Val Ile Asn Gln Ser Gln His Pro Val Ser
1 5 10 15
Ser Pro Ser Val
20

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2629 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGATTCCGTG ATGACACTAC ATCATGACTG ACAGTAAAAG CGATGGCCAG TTTTACAGTG 60
TGCAAGTGCC AGACTCAACT TTCAGTCTTC TAAACGTTA CCAGCAGTTG AAACCAATTG 120

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| | |
|--|------|
| GCTCTGGAGC CCAAGGAATT GTTTGTGCTG CTTTGTATAC AGTTCCTGGA ATAAATGTTG | 180 |
| CTGTCAAGAA GTTAAGTCGT CCTTTTCAGA ACCAAACGCA TGCAAAGAGA GCCTACCGTG | 240 |
| AAC TTGTCTT CCTAAAGTGT GTCAATCATA AAAATATAAT TAGCTTGTTA AATGTGTTCA | 300 |
| CACCACAAAA AACGCTAGAA GAATTCGAAG ATGTGTACTT GGTATGGAG TTAATGGAGC | 360 |
| CTAACTTATG TCAGGTTATT CATATGGAGC TGGACCATGA AAGAATGTCA TACCTCCTCT | 420 |
| ACCAGATGCT TTGTGGCATT AAGCACCTGC ATTCAGCTGG CATAATTCAT AGGGATTGA | 480 |
| AGCCTAGCAA CATTGTAGTA AATCAGACT GTACTCTCAA GATCCTTGAC TTTGGCCTGG | 540 |
| CACGGACAGC CTGTACCAAC TTTATGATGA CTCCTATGT GGTAACTCGC TACTATCGGG | 600 |
| CTCCAGAAGT CATCCTGGGC ATGGGCTACA AGGAGAAATGT GGACATCTGG TCTGTCGGGT | 660 |
| GCATCATGGC AGAAATGGTC CTCATAAAT CCTGTTCCCC AGGAAGAGAC TATATTGATC | 720 |
| AATGGAATAA AGTTATTGAA CAGCTAGGAA CACCATCGC AGAGTTCATC AAGAACTTC | 780 |
| AGCCAACTGT AAGGAATTAT GTGGAAAACA GACCAAAGTA CCCTGGAATC AAATTGAA | 840 |
| AGCTCTTTCC AGATTGGATA TTTCCTCAG AATCGAAGC AGACAAAATA AAAACAAGTC | 900 |
| AAGCCAGAGA TCTGTTATCG AAAATGTTAG TGATTGATCC GGACAAGCGG ATCTCTGTGG | 960 |
| ACGAAGCCTT GCGCCACCGG TATATTACTG TTTGGTATGA CCCCCTGAA GCAGAAGCGC | 1020 |
| CACCACCTCA AATTATGAT GCCCAGTTGG AAGAAAGAGA GCATGCGATT GAAGAGTGG | 1080 |
| AAGAACTAAT TTACAAAGAA GTGATGGACT GCGAAGAAAG AAGCAAGAAT GGGGTGAAAG | 1140 |
| ACCAGCCTTC AGATGCAGCA GTAAGCAGCA AGGCTACTCC TTCTCAGTGG TCATCCATCA | 1200 |
| ATGACATCTC ATCCATGTCC ACTGAGCACA CCCTGGCCTC AGACACAGAC AGCAGTCTCG | 1260 |
| ATGCCTCAAC CCGACCCCTG GAAGGCTGCC GATGAAACCT CGCAGATGCC GCACCTGTCT | 1320 |
| GTGAAGGACT CTGGCTTCCA TGGCCCTGAG CACATGGGAG CTGGTGGAA AATCAAGAA | 1380 |
| GCTCCATGTT CTGCATGTAA GAAACACGAC GCCTTGCCCC CACTCAGTTC CAGTAGGATT | 1440 |
| GCCTGCGTAG ACTGTAACAT GAGGCAGACG ATGTCTGGAG AAAAAGTACA AACCACACTG | 1500 |
| TTAGAAATTT TGTTCAGAT CATTCAAGTG AGCAATTAGA ATAGCCGAGT TCTTTTCAAG | 1560 |
| TGTTGTGGTG TCCTTGGTGA CAGATCATGT GTAACGTGG GGAATCGTAT GCATGTGACC | 1620 |
| ACAAATGCTT GCTTGAACCT GCCCATGTAG CACTTTGGGA ATCAGTATTT AATGCCAAA | 1680 |
| TAATCTTCCA GGTAGTTCTG CTCTAGAAAT AATCTCTTAA TCCTCTTTAG TAATTGGTG | 1740 |
| TCTGTCCACA AAAAAATAGA TTATGTGTGT ATGAATTGGC CACTATCATA TTATCATATT | 1800 |
| TTACCCACTT TTATGGTATG ATTTATCTG TCTTTGTAT TTCAGAAGCA ATATAATTAA | 1860 |
| ATTTATTTAA TAAATAAAAC TACAGCTTTT CTTAAATTTG TGATGTTTTA GCGTGAAGAT | 1920 |
| TACCACTGCT TTATATGAC ACTCTGTGTC CTTTAACTG CCCACTATGG GAAACTTTAC | 1980 |
| GTACAGCTTT CTGCATGACA AAGTTCCAAG TTGTATTTC CTCTGCTTAA CGACTTATGT | 2040 |
| CACCTTGAAT CCTGACCACA CATTTCTTTT TTCTGGTCC TCTGAACCTG GATCTAGAAT | 2100 |
| CCCTCAGAGA ACTTCACCTT CTTTATCACA AAGCACCCCA TCTCAGTAGA ATGAATCGGC | 2160 |

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AGATTCTGA GCCCCGCTGC CTAATGTAGA GCTGACAGGG TGGCTTCCCC AGAACGGTGG 2220
 GTGGGTGCAT CCTTCCCTGA GCCCACCCAT CCTTTGCTCC CCTCTCTTTA TTTAAGGTGA 2280
 AAGGTGATTG GGTCTCATAG CCTTTCCTTT TGTAGCATTG CCTAACTTGT CTTTCTCACT 2340
 GACAGAAGCC ACCACGTCCA GCCAGAGCAC ATGGTCTCTT AGGAGACCGG GCTTACTTAC 2400
 CATGCATGTT TGCTGCTGTC CTTTCCATT TTGTGGAGGC ATTTCCCTTTT TCTAAGGGAA 2460
 TTCTCAGAT GTTCTAGAAA CATTGAGAAG AACGCAGAAG AAATATTCTA GAGAATTGGG 2520
 GGTTCATTCT TGAATATTTT CTGATTAAA ACTGCTCACC TGAATTGAT ACTTTCAGAT 2580
 CCTGATCTTG TAAATTACTC GAGATTGCT AAGATGCTGA GTTCTCTGT 2629

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 2629 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGATTCTGT ATGACACTAC ATCATGAGTG ACAGTAAAAG CGATGGCCAG TTTTACAGTG 60
 TGCAAGTGCC AGACTCAACT TTCCTGTC TAAAACGTTA CCAGCAGTTG AAACCAATTG 120
 GCTCTGGAGC CCAAGGAATT GTTTGTGCTG CTTTGATAC AGTTCTTGA ATAAATGTTG 180
 CTGTCAAGAA GTTAAGTCGT CCTTTCAGA ACCAAACGCA TGCAAGAGA CCTACCGTG 240
 AACTTGTCCT CCTAAAGTGT GTCAATCATA AAAATATAAT TAGCTTGTTA AATGTGTTCA 300
 CACCACAAA AACGCTAGAA GAATCCAAAG ATGTGTAATT GGTATGAG TTAATGGACG 360
 CTAACCTATG TCAGGTTATT CATATGGAGC TGGACCATGA AAGAAATGCA TACCTCTCT 420
 ACCAGATGCT TTGTGGCATT AAGCACCTGC ATTCAGCTGG CATAATTCAT AGGGATTGTA 480
 AGCCTAGCAA CATTGTAGTA AAATCAGACT GTACTCTCAA GATCCTTGAC TTTGGCCTGG 540
 CACGGACAGC CTGTACCAAC TTTATGATGA CTCCTATGT GGTAACTGCG TACTATCGGG 600
 CTCCAGAAGT CATCTGGGGC ATGGGCTACA AGGAGAATGT TGATATCTGG TCAGTGGGTT 660
 GCATCATGGG AGAGCTGGTG AAAGGTTGTG TGATATTCCA AGGTACTGAC CATATTGATC 720
 AATGGAATAA AGTTATTGAA CAGCTAGGAA CACCATCCGC AGAGTTGATG AAGAACTTC 780
 AGCCAACTGT AAGGAATTAT CTGGAAAACA GACCAAAGTA CCCTGGAATC AAATTGAAAG 840
 AGCTCTTTCC AGATTGGATA TTTCGCTCAG AATCOGAACG AGACAAAATA AAAACAAGTC 900
 AAGCCAGAGA TCTGTTATCG AAAATGTTAG TGATTGATCC GGACAAGCGG ATCTCTCTGG 960
 ACGAAGCCTT GCGCCACCGG TATATTACTG TTTGGTATGA CCCCCTGAA GCAGAAGCGC 1020
 CACCACCTCA AATTTATGAT GCCCAGTTGG AAGAAAGAGA GCATGCGATT GAAGACTGGA 1080
 AAGAACTAAT TTACAAAGAA GTGATGGACT GCGAAGAAAG AAGCAAGAAT CGGGTGAAAG 1140

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| | |
|---|------|
| ACCAGCCTTC AGATGCAGCA GTAAGCAGCA AGGCTACTCC TTCTCAGTCG TCATCCATCA | 1200 |
| ATGACATCTC ATCCATGTCC ACTGAGCACA CCCTGGCCTC AGACACAGAC AGCAGTCTCG | 1260 |
| ATGCCTCAAC CGGACCCCTG GAAGGCTGCC GATGAAACCT CGCAGATGGC GCACCTGTCT | 1320 |
| GTGAAGGACT CTGGCTTCCA TGGCCCTGAG CACATGGGAG CTGGTGGAAC AAATCAAGAA | 1380 |
| GCTCCATGTT CTGCATGTAA GAAACAGAC GCCTTGCCCC CACTCAGTTC CAGTAGGATT | 1440 |
| GCCTGCGTAG ACTGTAACT GAGGCAGACG ATGTCTGGAG AAAAGTACA AACCACACTG | 1500 |
| TTAGAAATTT TGTTCAGAT CATTCAAGTG AGCAATTAGA ATAGCCGAGT TCTTTTCAAG | 1560 |
| TGCTGTGGTG TCCTTGGTGA CAGATCATGT GTAACGTGG GGAAGTATG GCATGTGACC | 1620 |
| ACAAATGCTT GCTTGAACCT GCGCATGTAG CACTTTGGGA ATCAGTATTT AAATGCCAAA | 1680 |
| TAATCTTCCA GGTAGTCTG CTCTAGAAAT AATCTCTTA TCCTCTTTAG TAATTTGGTG | 1740 |
| TCTGTCCACA AAAAATAGA TTATGTGTGT ATGAATTGGC EACTATCATA TTATCATATT | 1800 |
| TTACCCACTT TTATGGTATG ATTTATCTG TCTTTGTAT TTCAGAAGGA ATATAATTAA | 1860 |
| ATTTATTTAA TAAATAAAAC TACAGCTTTT CTAAATTTG TGATGTTTTA GGCTGAGAAT | 1920 |
| TACCACTGCT TTATATGAC ACTCTGTGC CTTTAAACTG CCCACTATGG GAAACTTTAC | 1980 |
| GTACAGCTTT CTGCATGACA AAGTTCCAAG TTGTATTICA CTCTGCTTAA CGACTTATGT | 2040 |
| CACCTTGAAT CCTGACCACA CATTTCCTTT TTCTTGGTCC TCTGAACITG GATCTAGAAT | 2100 |
| CCCTCACAGA ACTTCACCTT CTTTATCACA AAGCACCCCA TCTCAGTAGA ATGAATCGGC | 2160 |
| AGATTCTTGA GCGCCGCTGC CTAATGTAGA GCTGACAGGG TGGCTTCCCC AGAACGGTGG | 2220 |
| GTGGGTGCAT CCTTCCCTGA GCGCACCCAT CCTTTGCTCC CCTCTCTTAA TTTAAGGTGA | 2280 |
| AAGGTGATTG GGTCTCATAG CCTTTCCTTT TGTAGCATTG CCTAACTTGT CTTTCTCACT | 2340 |
| GACAGAAGCC ACCAGTCCA GCCAGAGCAC ATGGTCTCTT AGGAGACCGG GCTTACTTAC | 2400 |
| CATGCATGTT TGCTGCTGTC CTTTCCATT TTGTGGAGGC ATTTCTTTT TCTAAGGGAA | 2460 |
| TTCTCAGAT GTTCTAGAAA CATTGAGAAG AACGCAGAAG AAATATTCTA GAGAATTGGG | 2520 |
| GGTTCATTCT TGAATATTTT CTGATTTAAA ACTGCTCACC TGAATTGAT ACTTTCAGAT | 2580 |
| CCTGATCTTG TAAATTACTC GAGATTGGT AAGATGCTGA GTTCTCTGT | 2629 |

(2) INFORMATION FOR SEQ ID NO:11:

- (1) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1975 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:11:

| | |
|---|-----|
| CCCTCCTTAT TCCGGTTTGG AATGTGGCTA ATCAAAGCCC AGTAGGAGGA TTTCTGGGGC | 60 |
| AAACAGGTGG ACCAGGATCC TGGTTCTCAG GCACGGAATG GCTATTGTGA GAGCGCCACC | 120 |

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| | |
|--|------|
| AGCAGGACCA TCGCAGATCT TGGTTATGGC TGCTCAGCA AGAGGCTGTT GATGTAGACC | 180 |
| CCCTTTCCCG TAGATGAGAA ATCACAGGAG CAGTGGTATT TATGAGCCTC CATTTCCTAT | 240 |
| ACTACTGCAG TGAACCAACC TTGGATGTGA AAATTGCCTT TTGTCAGGTG TGTGTTCCCTT | 300 |
| ACAGGTAATA CAAAGGGATT CGACAAACAC GTGGATGTGT CTCTGTGTTT CAAACATTAC | 360 |
| AACATGAGCA AAAGCAAGGT AGATAACCAG TTCTACAGTC TGGAACTGGG AGACTCAACC | 420 |
| TTACAGGTC TAAAGCGCTA CCAGAACCTG AAGCCGATCG GCTCTGGGGC TCAGGGAATA | 480 |
| GTGTTGTGCTG CGTATGACGC TGTCTOGAC AGAAATGTGG CCATTAAGAA GCTCAGCAGA | 540 |
| CCCTTCAGCA ACCAACTCA TGCCAAGAGG GCTTACGGG AGCTGGTCCT CATGAAGTGT | 600 |
| GTGAACATA AAAACATTAT TAGCTTATTA AATGTCCTTA CACCCAGAA AACACTGGAG | 660 |
| GAGTTCCAAG ATGTTTACTT AGTGATGGAA CTGATGGAAG CCAACTGTG TCAGGTGATT | 720 |
| CAGATGGAGC TGGACCAAGA GCGGATGTG TACTTGCTGT ACCAGATGCT GTGGGGATC | 780 |
| AAACACCTCC ACTCGCTGG GATCATCCAC AGGGACTTAA AACCCAGTAA CATCGTAGTC | 840 |
| AAGTCTGATT GCACACTGAA AATCCTGGAC TTTGGACTGG CCAGGACAGC GGGCACAAGC | 900 |
| TTCATGATGA CTCGATATGT GGTGACGAGA TATTACAGAG CCCCCAGGT CATCCTGGGC | 960 |
| ATGGGCTACA AGGAGAAGT GCACATATGG TCTGTGGGCT GCATCATGGG AGAAATGGTT | 1020 |
| CGTCACAAA TOCTCTTCC CGGAAGGAC TATATTGACC AGTGAACAA AGTCATAGAG | 1080 |
| CAGCTAGGAA CTCGCTGTCC AGAATTCATG AAGAAATTGC AGCCACCGT CAGAACTAC | 1140 |
| GTGGAGAACC GGCCCAAGTA TGCAGGCCTC ACCTTCCCA AGCTCTTCC AGATTCCCTC | 1200 |
| TTCCAGCGG ATTCGAGCA CAATAAACTT AAAGCCAGCC AAGCCAGGA CTGTTGTCA | 1260 |
| AAGATGTTAG TGATTGACCC AGCGAAGAGG ATATCGGTGG ATGACGCATT GCAGCATCCG | 1320 |
| TACATCAAG TTTGGTAAGA CCTGCTGAA GTGGAGGCGC CTCGGCTCA GATATATGAC | 1380 |
| AAGCAATTGG ATGAAAGGGA GCACACCATC GAAGAATGGA AAGAACTCAT CTACAAGGAA | 1440 |
| GTAATGAAC CAGAAGAGAA GACTAAGAAC GCGTAGTCA AAGCCAGCC CTCACCTTCA | 1500 |
| GGTGACAGC TGAACAGCAG TGAGAGTCTC CCTCCATCCT CATCTGTCAA CGACATCTCC | 1560 |
| TCCATGTCCA CCGACCAGAC CCTGCGATCC GACACTGACA GCAGCCTGGA AGCCTCGGG | 1620 |
| GGACCGCTGG GTTGTTCAG GTGACTAGCC GCGTGCCTGC GAAACCCAGC GTTCTTCAGG | 1680 |
| AGATGACGCC ATGATAGAAC ACAGGCCACA TGCACACACA CAGAGCTTGT ACACACACAC | 1740 |
| ACACACACAC ACACACCCAC GCACGCCAGC ACGCAAGCAC GCACGCCAGC ACAAATGCAC | 1800 |
| TCACGCAATG TCAAGAAAA AAAAAGTAGC GAGAGAGAGC GAGAGAGCCA ACGTAAACT | 1860 |
| AAGTTAAATC TTTCTGGTG CTCTCCAGA GTTCTGTATC GCAGCTGAGC TGAATGTAT | 1920 |
| ACTTAACTTC TAGTCGGCT CGCTCGACTT TGCTCTCCCT CCGGCAGTGC TTAAT | 1975 |

(2) INFORMATION FOR SEQ ID NO:12:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1986 base pairs

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

| | |
|--|------|
| CCCTATCCCT CCTTATCCG GTTGGAAATG TGGCTAATGA AAGCCAGTA GGAGGATTTC | 60 |
| TGGGGCAAAC AGGTGGACCA GGATCCTGGT TCTCAGGCAC GGAATGGCTA TTGTGAGAGC | 120 |
| GCCACCAGCA GGACCATCCG AGATCTTGGT TATGGCTGCT CACGCAAGAG GCTGTTGATG | 180 |
| TAGACCCCTT TTCCCGTAGA TGAGAAATCA CACGAGCAGT GGTATTTATG AGCCTCCATT | 240 |
| TCTTATACTA CTGCAGTGAA CCAACCTTGG ATGTGAAAAT TGCCFTTTGT CAGGTGTGTG | 300 |
| TTCCCTTACG GTAAACAAA GCGATTGGAC AAACACGTGG ATGTGTCTTC TGTGTCAAA | 360 |
| CATTACAACA TGAGCAAAAG CAAGGTAGAT AACCACTTCT ACAGTGTGGA AGTGGGAGAC | 420 |
| TCAACCTTCA CAGTTCTAAA GCGCTACCAG AACCTGAAGC CGATCGGCTC TGGGGCTCAG | 480 |
| GGAATAGTTT GTGCTGGTA TGACGCTGTC CTGACAGAA ATGTGGCCAT TAAGAAGCTC | 540 |
| AGCAGACCCT TCCAGAACCA AACTCATGCC AAGAGGGCTT ACCGGGAGCT GGTCTCATG | 600 |
| AAGTGTGTGA ACCATAAAAA CATTATTAGC TTATTAAATG TCTTTACACC CCAGAAAAACA | 660 |
| CTGGAGGAGT TCCAAGATGT TTACTTAGTG ATGGAATGTA TGGACGCCAA CTTGTGTGAG | 720 |
| GTGATTGAGA TGGAGCTGGA CCAAGAGCGG ATGTGCTACT TGCTGTACCA GATGCTGTGG | 780 |
| GCGATCAAAC ACCTCCACTC CGCTGGGATC ATCCACAGGG ACTTAAACC CAGTAACATC | 840 |
| GTAGTCAAGT CTGATTGCAC ACTGAAAATC CTGACTTTG GACTGGCCAG GACAGCGGGC | 900 |
| ACAAGCTTCA TGATGACTCC GTATGTGGTG ACGAGATATT ACAGAGCCCC CGAGGTCATC | 960 |
| CTGGGCATGG GCTACAAGGA GAACTGGAC ATATGGTCTG TGGGCTGCAT CATGGGAGAA | 1020 |
| ATGGTTGCTC ACAAATCCT CTTTCCCGCA AGGGACTATA TTGACCAAGT GAACAAAGTC | 1080 |
| ATAGAGCAGC TAGGAATCC GTGTCCAGAA TTCATGAAGA AATTGCAGCC CACCGTCAGA | 1140 |
| AAGTACGTGG AGAACCGGCC CAAGTATGCA GGCCTCACCT TCCCAAGCT CTTTCCAGAT | 1200 |
| TCCCTCTTCC CAGCGGATTC CGAGCACAAT AAACCTAAAG CCAGCCAAGC CAGGGACTTG | 1260 |
| TTGTCAAAGA TGTTAGTGAT TGACCCAGCG AAGAGGATAT CGGTGGATGA CGCATTGCAG | 1320 |
| CATCGGTACA TCAACGTTG GTACGACCT GCTGAAGTGG AGGCGCTCC GCCTCAGATA | 1380 |
| TATGACAAGC AATTGGATGA AAGGGAGCAC ACCATCGAAG AATGGAAAGA ACTCATCTAC | 1440 |
| AAGGAAGTAA TGAATCAGA AGAGAAGACT AAGAACGGCG TAGTCAAAGG CCAGCCCTCA | 1500 |
| CCTTCAGCAC AGGTGCAGCA GTGAACAGCA GTGACAGTCT CCCTCCATCC TCATCTGTCA | 1560 |
| AAGACATCTC CTCCATGTCC ACCGACCAGA CCCTCGCATC CGACACTGAC AGCAGCCTGG | 1620 |
| AAGCCTCGGC GGGACCGCTG GGTGTGTGCA GGTGACTAGC CGCTGCGCTG CGAAACCCAG | 1680 |
| CGTTCTTCAG GAGATGACGC CATGATAGAA CACAGCGCAC ATGCACACAC ACAGAGCTTG | 1740 |

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| | |
|--|------|
| TACACACAC'. CACACACACA CACACACGCA CGCAGGCACG CAGGCAAGCA CGCAGGCACG | 1800 |
| CACAAATGCA CTCACGCAAT GTCAAGAAAA AAAAAAGTAG CGAGAGAGAG CGAGAGAGCC | 1860 |
| AACGTAAAAAC TAAGTTAAAT CTTTCTGCGT GCTTCTCCAG AGTTCTGTAT CGCAGCTGAG | 1920 |
| CTGAAATGTA TACTTAACTT CTAGTCGGCG TCGCTCGACT TTGGTCTCCC TCCGGCAGTG | 1980 |
| CTTACT | 1986 |

(2) INFORMATION FOR SEQ ID NO:13:

- (1) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1408 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:13:

| | |
|--|------|
| GCGGCGGAGC GCGGGAAGTT GCGGCGGAAA GCGGAGCGCG CGAGCAGGAT TAAGTAGCGG | 60 |
| CCGGCGCACC GGCACGGGCG CGCTCTCCGC TACTGGCTTC CAGGTCTCCG TTGGCTGCAC | 120 |
| TGCGGCGCGG TTGTTGAATA TTTGGATGAA GCCATTAGAC TAATTGCTTG CCATCATGAG | 180 |
| CAGAAGTAAA CGTGACAACA ATTTTATAG TGTAGAGATC GCAGATTCTA CATTACAGT | 240 |
| CCTAAAACGA TACCAGAACT TAAAGCCTAT AGGCTCAGGA GCTCAAGGAA TAGTGTGTGC | 300 |
| AGCTTATGAT GCTATTCTTG AAAGAAATGT TCCAATCAAG AAGCTCAGCC GGCCATTTC | 360 |
| GAATCAGACC CATGCTAAGC GAGCCTACCG AGAAGTAGTT CTTATGAAGT GTGTTAATCA | 420 |
| CAAAAATATA ATTGGCCTTT TGAATGTTT CACACCACAG AAATCCCTAG AAGAATTTC | 480 |
| AGATGTTTAC ATAGTCATGG AGCTCATGGA TGCAATCTT TGCCAAGTGA TTCAGATGGA | 540 |
| GTTAGATCAT GAAAGAATGT CCTACCTTCT CTATCAATG CTGTGTGGAA TCAAGCACCT | 600 |
| TCACTCTGCT GGAATTATTC ATCGGGACTT AAAGCCTAGT AATATAGTAG TCAATATAGA | 660 |
| CTGCACCTTG AAGATTCTTG ATTTTGGACT GGCAAGGACT GCAGGAACGA GATTCTTGAT | 720 |
| GACGCCITAC GTGGTAACTC GTTACTACAG AGCACCAGAG GTCATTCTCC GCATGGGCTA | 780 |
| CAAGGAGAAC GTGGATTAT GGTCTGTGGG GTCCATTATG GGAGAAATGG TTTGCTCAA | 840 |
| AATCCTCTTT CCAGGAAGGG ACTATATTGA TCAGTGGAAAT AAAGTTATTG AACAGCTCGG | 900 |
| AACACCTTGT CCTGAATTCA TGAAGAACT ACAACCAACA GTAAGGACTT ACGTTGAAAA | 960 |
| CAGACCTAAG TAGCTGGCT ATAGCTTTGA GAAACTGTTT CCTGATGTGC TTTTCCAGC | 1020 |
| TGACTCAGAA CATAACAAAC TTAAGGCCAG TCAGGCGAGA GATTGTAT CTAAAAATGCT | 1080 |
| GGTGATAGAT GCGTCCAAA GATCTCGT AGACGAAGCT CTCCAGCACC CGTACATCAA | 1140 |
| CGTCTGTAT GATCCTTCAG AAGCAGAGGC CCCACCACCA AAGATCCCTG ACAAGCACTT | 1200 |
| AGATCAAAGG GAGCACACAA TAGAGGAGTG GAAAGAATG ATATACAAGG AGGTCAATGA | 1260 |
| TTTGAGGAGG CGAACTAAGA ATGCGTCAT AAGAGGGCAG CCGTCTCCTT TAGGTGCAGC | 1320 |

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AGTGATCAAT GGCTCTCAGC ATCCGGTCTC TTCGGCGTCT GTCAATGACA TGTCTTCAAT 1380
GTCCACAGAT CCGACTCTGG CCTCGGAT 1408

(2) INFORMATION FOR SEQ ID NO:14:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCAGATG CAGTAAGCAG CAAGGCTACT CCTTCTCAGT CGTCATCCAT C 51

(2) INFORMATION FOR SEQ ID NO:15:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 63 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CAGCCCTCAC CTCAGGTGC AGCAGTGAAC AGCAGTGAGA GTCTCCCTCC ATCCTCATCT 60
GTC 63

(2) INFORMATION FOR SEQ ID NO:16:

- (1) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 60 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(11) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCTCCTTTAG GTGCAGCACT GATCAATGCC TCTCAGCATC CGGTCTCTTC GCCGTCTGTC 60

What is claimed is:

1. A recombinant p54 stress-activated protein kinase polypeptide or biologically active fragment thereof at least 10 amino acids in length.

2. The polypeptide of claim 1, wherein said
5 polypeptide or fragment thereof is derived from an amino acid sequence substantially identical to SEQ ID NO: 1.

3. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence at least 95% identical to SEQ ID NO: 1.

10 4. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence substantially identical to SEQ ID NO: 2.

5. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino
15 acid sequence at least 95% identical to SEQ ID NO: 2.

6. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence substantially identical to SEQ ID NO: 3.

7. The polypeptide of claim 1, wherein said
20 polypeptide or fragment thereof is derived from an amino acid sequence at least 95% identical to SEQ ID NO: 3.

8. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence substantially identical to SEQ ID NO: 4.

25 9. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence at least 95% identical to SEQ ID NO: 4.

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10. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence substantially identical to SEQ ID NO: 5.

5 11. The polypeptide of claim 1, wherein said polypeptide or fragment thereof is derived from an amino acid sequence at least 95% identical to SEQ ID NO: 5.

12. The polypeptide of claim 1 wherein said polypeptide is derived from a mammal.

10 13. The polypeptide of claim 12 wherein said mammal is a rat.

14. The polypeptide of claim 12 wherein said mammal is a human.

15 15. The polypeptide of claim 1 wherein said polypeptide or fragment thereof is useful for producing antibodies which specifically bind to a p54 stress-activated protein kinase.

16. The antibody of claim 15 wherein said polypeptide fragment is chosen from the group consisting of SEQ ID NOS: 6, 7, and 8.

20 17. A DNA and its degenerate variants which encode a p54 stress-activated protein kinase polypeptide, or a biologically active fragment thereof at least 30 nucleotides in length.

25 18. The DNA of claim 17 comprising a nucleotide sequence encoding a p54 α I polypeptide at least 90% identical to SEQ ID NO 9.

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19. The DNA of claim 17 comprising a nucleotide sequence encoding a p54 α II polypeptide at least 90% identical to SEQ. ID NO.: 10.

20. The DNA of claim 17 comprising a nucleotide sequence encoding a p54 β I polypeptide at least 90% identical to SEQ. ID NO.: 11.

21. The DNA of claim 17 comprising a nucleotide sequence encoding a p54 β II polypeptide at least 90% identical to SEQ. ID NO.: 12.

22. The DNA of claim 17 comprising a nucleotide sequence encoding a p54 γ polypeptide at least 90% identical to SEQ. ID NO.: 13.

23. The DNA of claim 17 wherein said fragment is chosen from the group consisting of SEQ ID NOS: 14, 15, and 16.

24. The DNA of claim 17 wherein said DNA is derived from a mammal.

25. The DNA of claim 24 wherein said mammal is a rat.

26. The DNA of claim 24 wherein said mammal is a human.

27. A DNA which hybridizes under stringent conditions to one or more of the DNAs chosen from the group SEQ ID NO. 9, 10, 11, 12, and 13.

28. A method of screening potentially therapeutic compounds comprising the steps of

a) treating cultured cells by applying said compounds and stress-activated protein kinase-activating stimuli,

b) preparing cytoplasmic extracts of said treated
5 cells, then

c) assaying the isolated recombinant stress-activated protein kinases for c-Jun kinase activity.

29. The method of claim 28 wherein said cultured cells are selected from the group consisting of human
10 cell lines HepG2, CCD-18Co, U937, and HT-29.

30. A method of screening potentially therapeutic compounds comprising the steps of

a) treating cultured cells by applying said compounds,

15 b) preparing cytoplasmic extracts of said treated cells,

c) combining said extracts with an inactive recombinant stress-activated protein kinase, and then

d) assaying the isolated recombinant stress-
20 activated protein kinases for c-Jun kinase activity.

31. The method of claim 30 wherein said cultured cells are selected from the group consisting of human cell lines HepG2, CCD-18Co, U937, and HT-29.

32. The method of claim 30 wherein said cultured
25 cells have been treated previously or concurrently with stress-activated protein kinase-activating stimuli.

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FIG. 1

MSDEKSDQFYSVQVADSTFTVLKRYCCLPISGSAQGINCAAFDTVLGDTWAVVFLSRP p54aI
 MSDEKSDQFYSVQVADSTFTVLKRYCCLPISGSAQGINCAAFDTVLGDTWAVVFLSRP p54aII
 MSREKFDINIFYSEVQADSTFTVLKRYONLKPISGSAQGINCAAYDALLERNVAIKKLSRP p54yI
 MSREKFDINIFYSEVQADSTFTVLKRYONLKPISGSAQGINCAAYDALLERNVAIKKLSRP p54yII
 MSKSKNDONOFYSVQVADSTFTVLKRYONLKPISGSAQGINCAAYDALLERNVAIKKLSRP p54BI
 MSKSKNDONOFYSVQVADSTFTVLKRYONLKPISGSAQGINCAAYDALLERNVAIKKLSRP p54BII

FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKTLEEFQDVYLVMELMDANLCQVIR p54aI
 FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKTLEEFQDVYLVMELMDANLCQVIR p54aII
 FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKSLLEEFQDVYLVMELMDANLCQVIR p54yI
 FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKTLEEFQDVYLVMELMDANLCQVIR p54yII
 FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKTLEEFQDVYLVMELMDANLCQVIR p54BI
 FQNOTHAKRAYRELVLKCVNHKNIIISLLNVFTPOKTLEEFQDVYLVMELMDANLCQVIR p54BII

MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54aI
 MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54aII
 MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54yI
 MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54yII
 MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54BI
 MELDHERMSYLLYQMLCGIKHLHSAGIIRDLKPSNIVVMSDCTLKILDFGLARTAGTSF p54BII

MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54aI
 MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54aII
 MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54yI
 MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54yII
 MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54BI
 MYTFYVATRYYPAPFVILONGYKENTLWSVGCIMAGENVCKKILFFGRDYIDQWNVIEO p54BII

LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54aI
 LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54aII
 LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54yI
 LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54yII
 LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54BI
 LGTPESEFMYKLOPTVRYNRYENRKYVPGIKFEELFPDWIFPSESEEDKIKTSQARDLLSK p54BII

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MLVDPFPRISVDEAL RHPYITWYDPAEAEAPPPQIYDAQLEEREHAESEWKELIYKEV p54aI
MLVDPFPRISVDEAL RHPYITWYDPAEAEAPPPQIYDAQLEEREHAESEWKELIYKEV p54aII
MLVDPASKRISVDEALQHPYINWYDPAEAEAPPPQIYDKOLDERENTIESEWKELIYKEV p54YI
MLVDPFAKRISVDDALQHPYINWYDPAEAEAPPPQIYDKOLDERENTIESEWKELIYKEV p54YII
MLVDPFAKRISVDDALQHPYINWYDPAEAEAPPPQIYDKOLDERENTIESEWKELIYKEV p54YIII

MDWEERSKNGV-KDQPSD--AAV-SSKATPSQSSSINDISSMSTEHTLASDTSSSLDAST p54aI
MDWEERSKNGV-KDQPSD--AAV-SSKATPSQSSSINDISSMSTEHTLASDTSSSLDAST p54aII
MDLEERTKNGVIRGQPSPLGAAVINGSQHPVSSPSVNDSSHSTDOTLASDTSSSLEAAA p54YI
MNSEKTKNGVVKGQSPSPGAAVHSSSLP-FSSSVNDISSHSTDOTLASDTSSSLEASA p54YII
MNSEKTKNGVVKGQSPSPGXGAAY pII SEQ ID NO: 4

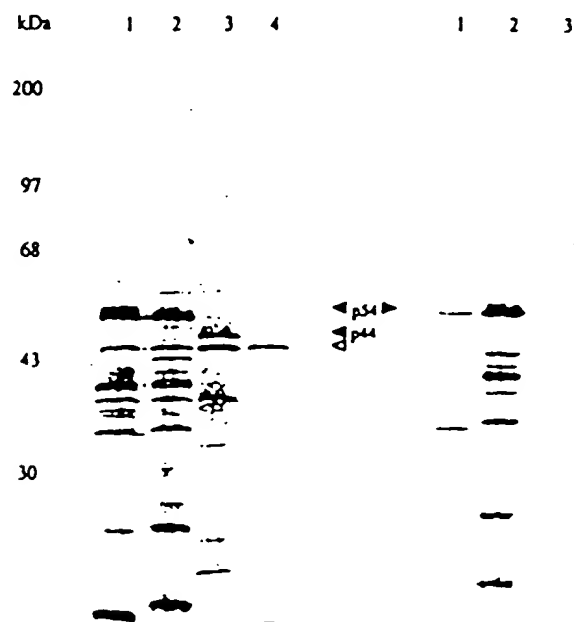
GPLEGCR p54aI SEQ ID NO: 1
GPLEGCR p54aII SEQ ID NO: 2
GPLGCCR p54YI SEQ ID NO: 3
GPLGCCR p54YII SEQ ID NO: 4

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FIG. 2A

FIG. 2B



Immunodepletion of p54s (SAPKs) removes >60% of GST-c-Jun kinase from extracts of TNF- α -treated Hep G2 cells

FIG. 3A

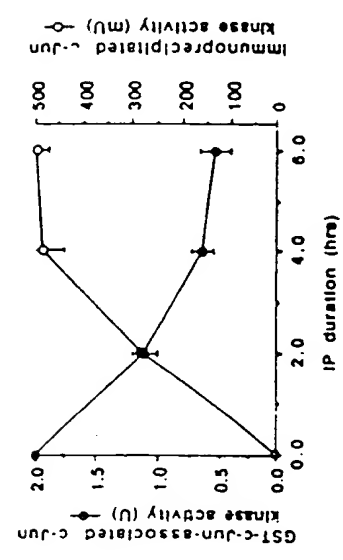
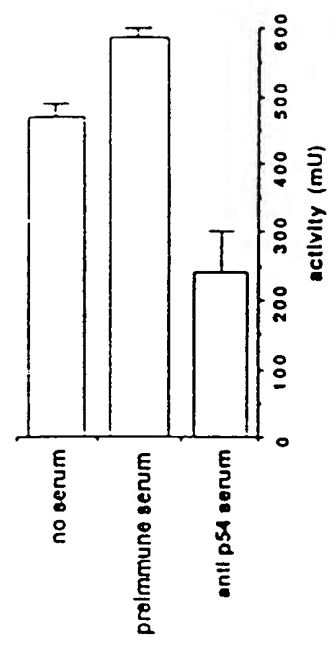


FIG. 3B



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FIG. 4A

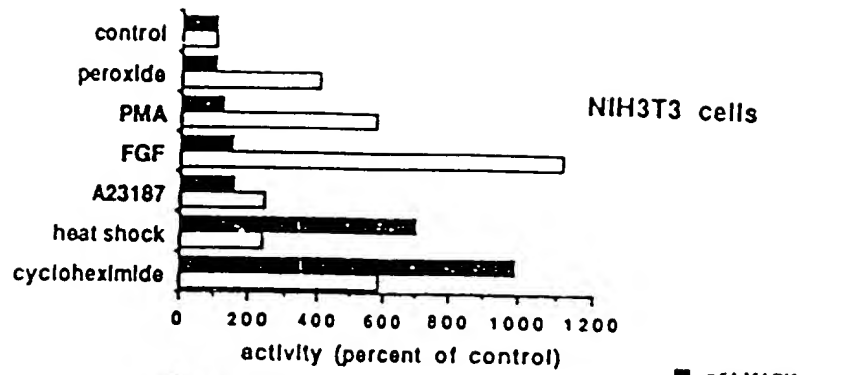
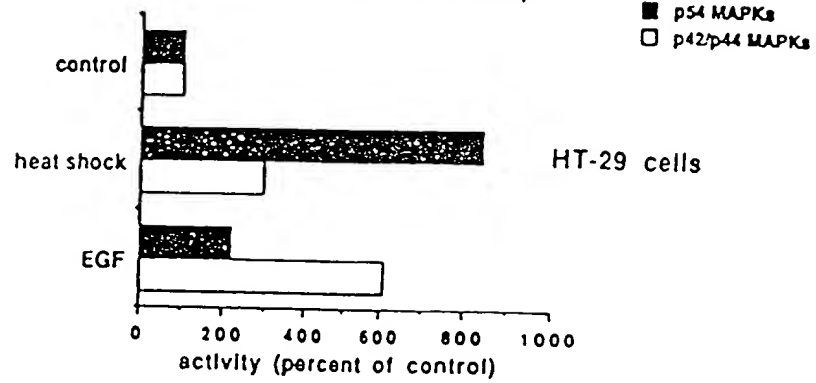


FIG. 4B



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FIG. 5A

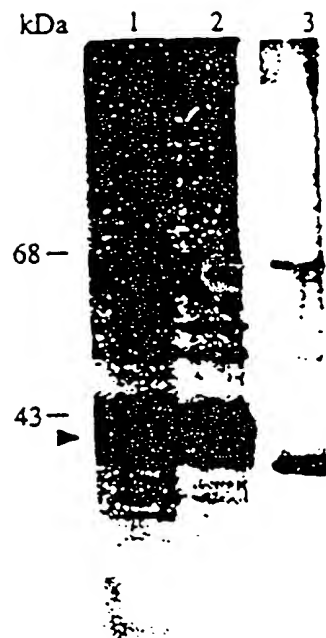


FIG. 5B

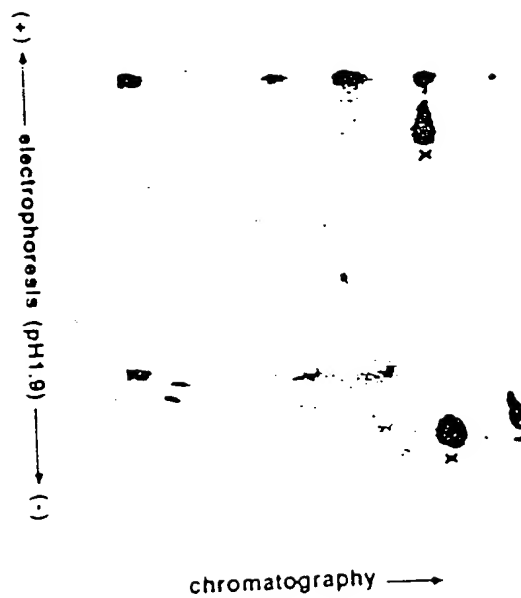


FIG. 6B

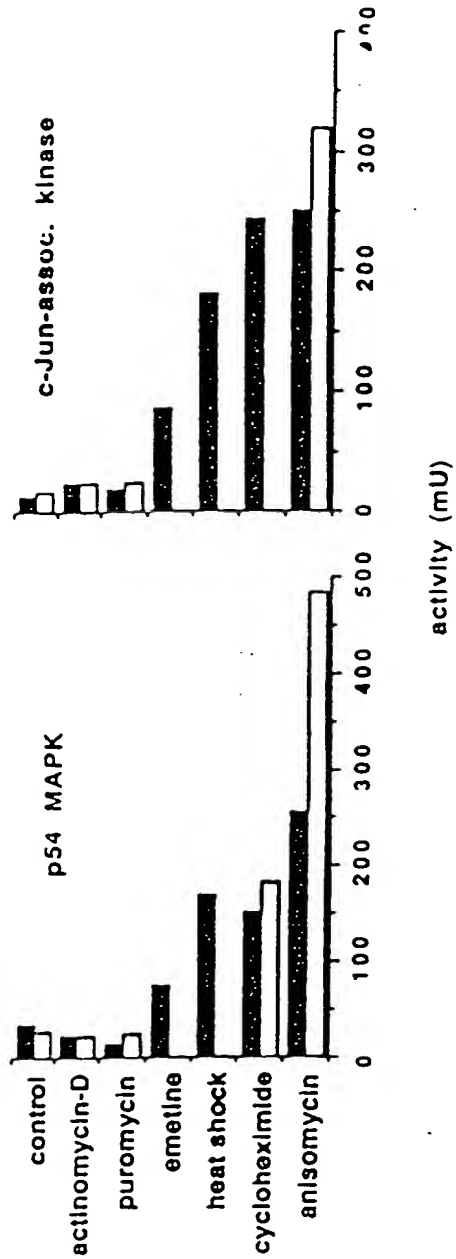
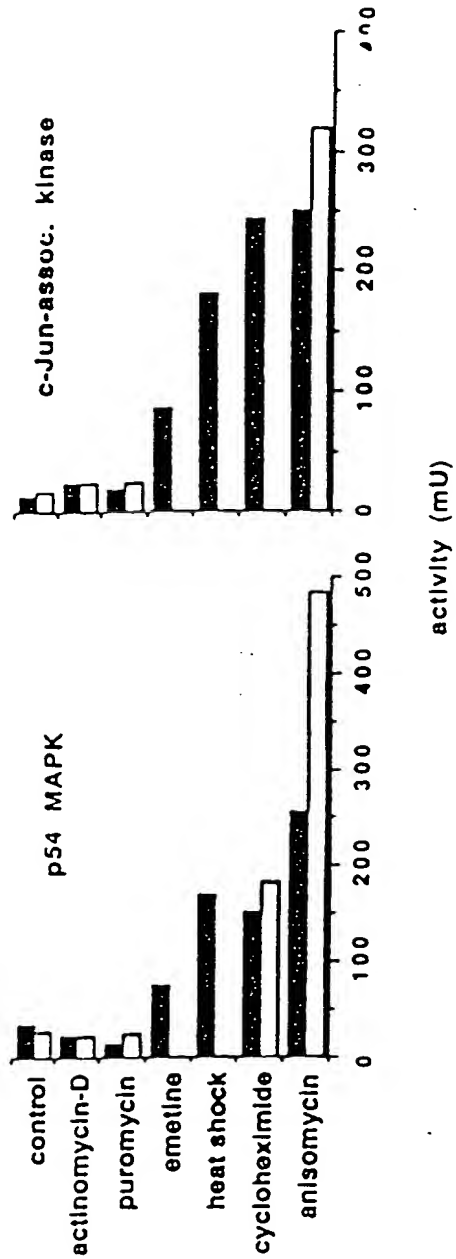


FIG. 6A



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FIG. 7A

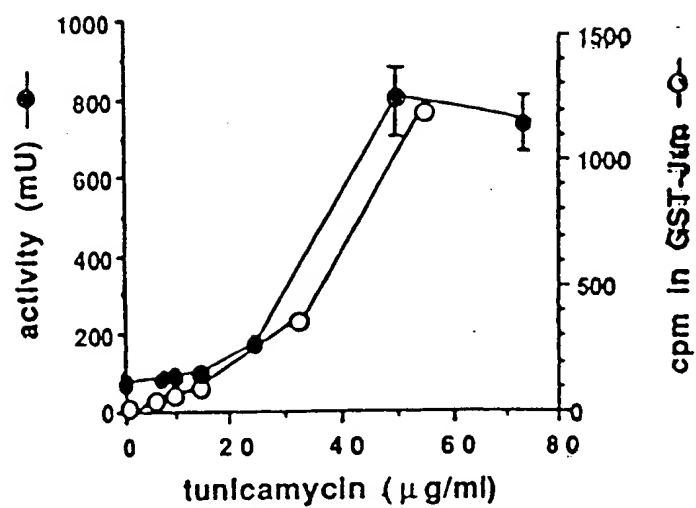
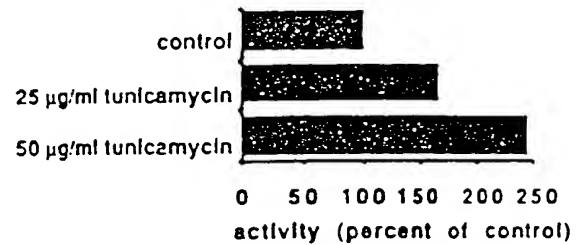


FIG. 7B



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FIG. 8A

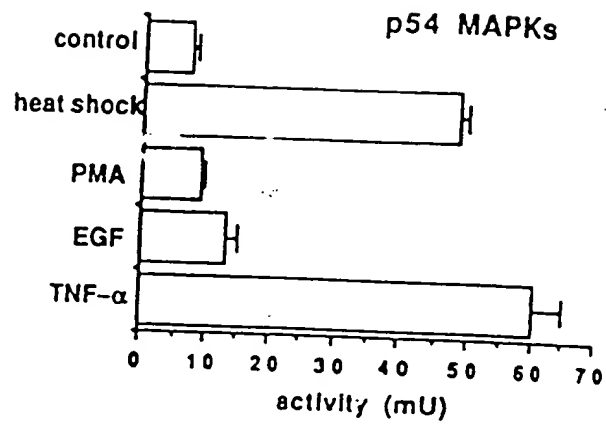
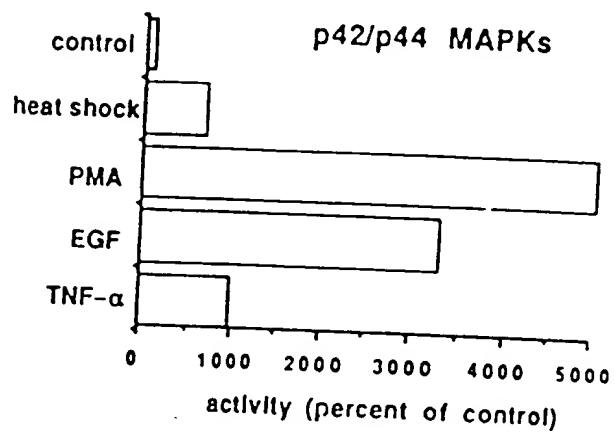


FIG. 8B



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FIG. 9A

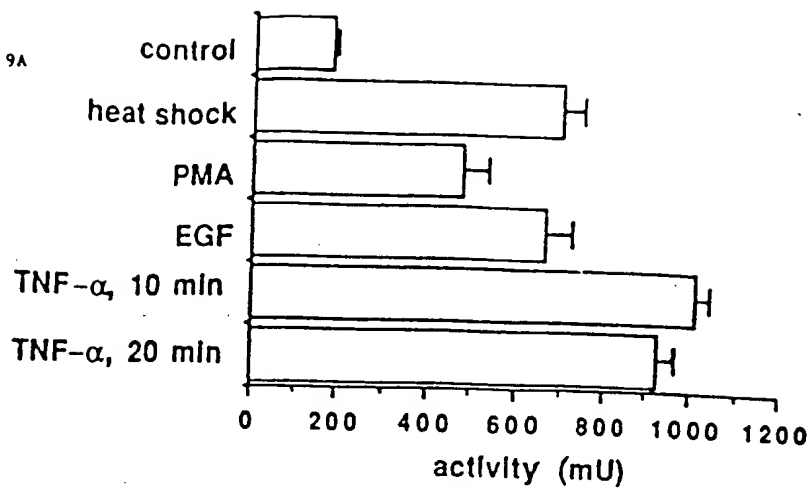
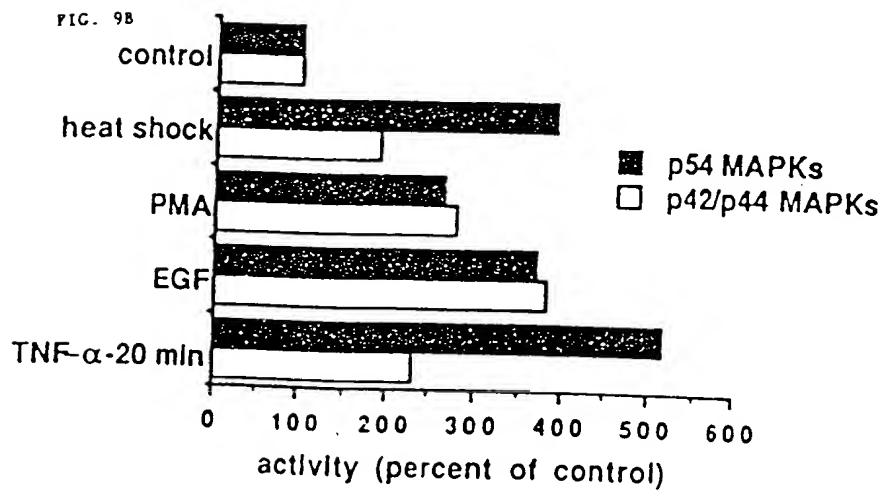
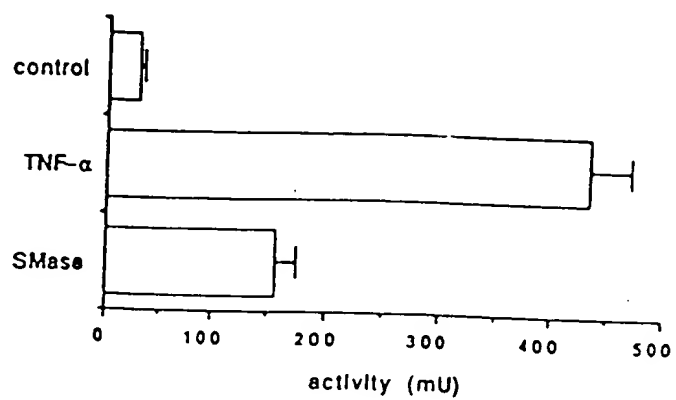


FIG. 9B



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FIG. 10



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Effect of IL-1- β on EL-4 cell MAPKs

FIG. 11A

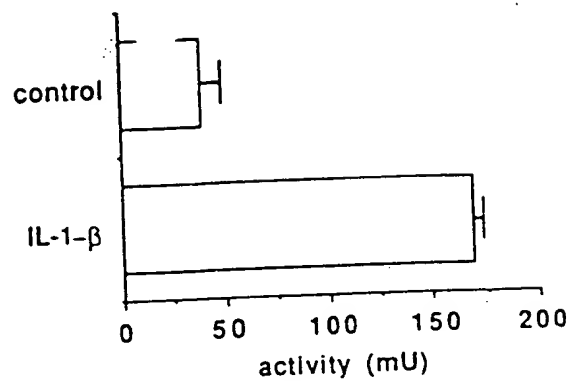
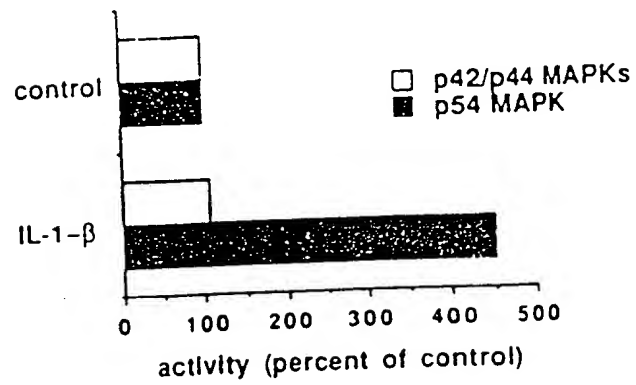


FIG. 11B



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FIG. 12A

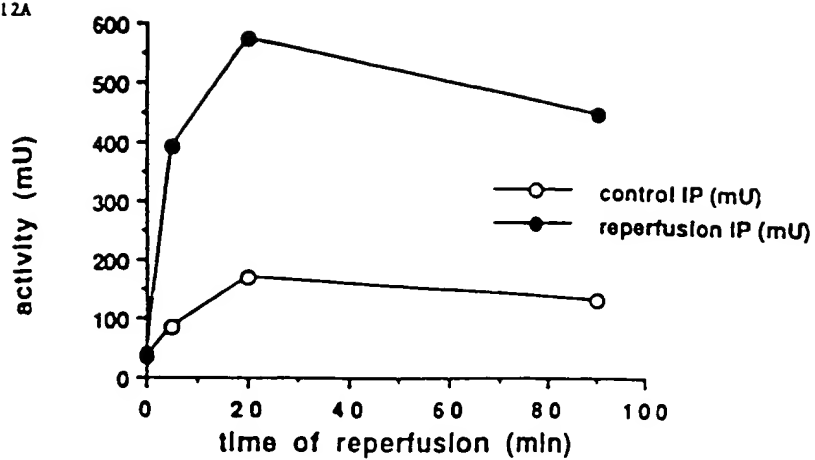
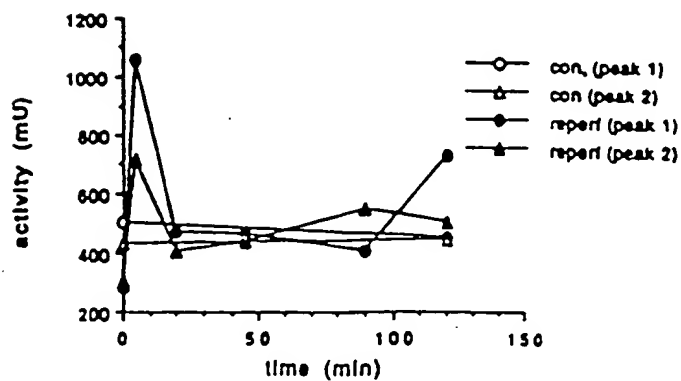


FIG. 12B



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FIG. 13 2148898

Activation of rat GST-SAPK- α by extracts of TNF-treated HepG2 cells

